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A Protocol for DNA Extraction from Feces of the Wild Pygmy Lorises, *Nycticebus pygmaeus*

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Abstract.- We developed a new method to isolate DNA from wild pygmy lorises' feces. The feces were washed two or three times with precooled acetone, which removed numerous potential PCR inhibitors. The DNA was purified with phenol/chloroform after incubation with proteinase K. D-loop region and cytochrome *b* were amplified and sequenced successfully from the extracted DNA. Comparison with the PCR products demonstrated the fecal DNA extracted by the improved protocol was better than the fecal DNA extracted without acetone preprocessing.

Keywords: Pygmy lorises, *Nycticebus pygmaeus*, fecal DNA, DNA extraction, noninvasive sampling.

INTRODUCTION

Two families of Lorises, *Nycticebus* (Primates: Prosimii: Loridae) are currently recognized – slow lorises (*Nycticebus coucang* and *Nycticebus bengalensis*) and pygmy lorises (*Nycticebus pygmaeus*). *N. pygmaeus* has a much more limited distribution range than the slow lorises (Wolfhein, 1983). Its current diversity is in mainland Southeast Asia, from China to Vietnam. These are found only in Pingbian, Hekou, Maguan, Jinping and Luchun of Yunnan, near the Sino-Vietnamese border (Wang, 1998; Zhang *et al*., 2002; Fooden, 1996; Duckworth, 1994). *N. pygmaeus* is a nocturnal and almost entirely arboreal primate. It typically lives in semi-evergreen and secondary forests (Wolfhein, 1983; Polet *et al*., 2004; Groves, 1970). The pygmy lorises' food diversity include gums, fruits, birds' eggs, chicks, geckos and arboreal small mammals (Ratajszczak, 1998; Tan, 1994), but it is an insectivorous species. *N. pygmaeus* is one of the least studied species of all prosimians (Fitch-Snyder and Jurke, 2003). The Appendix II of the Convention on International Trade in Endangered Species (CITES) included pygmy lorises (Wang, 1998). In its latest assessment in 2006 the IUCN classified the pygmy lorises as ''vulnerable" (IUCN). It is also listed as one of the first protected animals in China. Therefore, new field research methods appropriate for these primates are urgently required for the study of the pygmy loris.

Noninvasive samples of animals such as feces, hair and feathers are commonly collected for studies of wildlife population (Dalèn *et al*., 2004; Hogan *et al*., 2008). The DNA extracted from noninvasive sources has been developed and tested on a wide range of species. The noninvasive genetic surveys found broad application for conservation genetics and wildlife management, in especial as species that are endangered, elusive, or exist at low densities over vast areas (Kohn *et al*., 1999; Adams *et al*., 2003; Eggert *et al*., 2003; Wilson *et al*., 2003; Bellemain *et al*., 2005; Prugh *et al*., 2005; Smith *et al*., 2005). Pygmy lorises, the risk species.

The fecal DNA genotyping of is more advantageous than the traditional live-trapping survey technique (Taberlet and Luikart, 1999; Fernando *et al*., 2003; Smith *et al*., 2005). The objective of this study was to establish an extraction method for DNA from fecal samples, for conducting non-invasive surveys.

MATERIALS AND METHODS

Fecal samples

Fecal samples from seven individuals of pygmy loris were collected from the Daweishan

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Nature Reserve of Pingbian, Yunnan Province, China. Samples of fresh fecal material were transported to the laboratory in an icebox, and stored at –70°C. The procedure in this study followed protocols approved by China Wildlife Conservation Association, American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates, and also legal requirements of China.

Total DNA isolation

DNA was extracted by three different methods *viz.*, alcohol extraction method (Zhang *et al*., 2004), PBS extraction (Sambrook and Russell, 2002) and acetone extraction method (Zhong *et al*., 2003). The method ultimately developed is described below:

1) The fecal sample (0.05-0.10 g) was placed in a 2.0 ml centrifuge tube, to which double distilled water was added. The sample was mixed thoroughly and then kept at room temperature for 2 min.
2) The supernatant was poured in a 2.0 ml centrifuge tube, and centrifuged at 4,500x g at 4°C for 4 min;
3) The supernatant solution was once again poured in another 2.0 ml centrifuge tube, and centrifuged at 5,040 g at 4°C for 6 min to pellet down the cells.
4) Supernatant was discarded and 500µl pre-cooled (at -20°C) acetone was added in the cells, swirled and then after one minute centrifuged at 5,040 g at 4°C for 5 min. The cell pellet was washed with acetone, twice or three times.
5) The acetone was poured out and then the cells were treated with 500µl lytic buffer (200 mM NaCl, 100 mM Tris-HCl pH 8.0, 2.0% SDS, 50 mM EDTA, 1.0% Triton X-100) at 55°C for 3 h or 24 h. Then added 1.25 µl RNase (10mg/ml) at 37 °C for 30 min.
6) To the above lysate equal volume of mixture of phenol: chloroform: isoamyl alcohol (25:24:1 volume), was added then mixed and kept for 1 min, and centrifuged at 16,000 g at 4°C for 10 min, The supernatant was collected and the pellet was wash by phenol: chloroform: isoamyl alcohol (25:24:1) 2 to 3 times.
7) The above supernatant containing DNA was treated with equal volume of isoamyl alcohol at -20°C for 12 h
8) The DNA was centrifuged at 16,000 g at 4°C for 2 min. The supernatant was discarded while the pellet was washed with 70% ethanol and then with 20µl double distilled water twice. The DNA was stored at -20°C.

Conventional electrophoresis was then performed to verify that DNA had remained intact (0.8% agarose gel, 100 volts, 0.5X TBE buffer) (Suau *et al*., 1999).

PCR

Polymerase chain reaction (PCR) amplification of *Cyt b* and D-loop was performed using primers shown below. Reactions were performed in 50 µl volume containing 1.0 µl DNA template, 5.0 µl 10 × PCR buffer (containing 25 mmol/l Mg^{2+}), 1.0 µl of dNTPs (10×10^{-3} mmol/ml), 2.0 µl primer (10×10^{-6} mmol/ml), 0.5µl *Taq* DNA polymerase, and 40.5 µl ddH_2O. Amplification conditions were as follows: 94°C initial denaturation for 5min; 30 cycles each of 94°C for 1min, 48 to 55°C for 1min, and 72°C for 1min; 72°C extension for 10min. PCR products were visualized on an agarose gel electrophoresis.

Cyt b	
sense	AGA CTC ATG ACT AAC ATT CGA
antisense	TAG GGC TGT GTC TTC ATT TGA G
D-loop (Chen *et al*., 2006)	
sense	CCT GAA GTA GGA ACC AGA TG
antisense	CTC CAC CAT GAG TAG CAC CCA AAG C

Sequencing and sequence analysis

PCR product was sequenced at the Beijing Genomics Institute (BGI). All the sequences was compared with the sequences in the Gen-Bank database using BLAST program available for the NCBI internet website (http://www.ncbi.nlm.nih.gov.).

Nucleotide sequence accession numbers

The DNA sequences from this study were submitted to GenBank under accession numbers: Cyt *b* HQ842857-HQ842862 and D-loop HQ842864-HQ842879.

RESULTS

Faecal DNA quality

There was the tailing phenomenon in DNA by the different extract methods; all were different degree of degradation (Fig. 1); especially, the alcohol extract method and the PBS extract method; the products of the alcohol extract method and the PBS extract method were more segments, which were less than 100bp; however, the product of the acetone extract method were long fragments more than 2000bp and less degradation. The A260/A280 was about 1.8-2.0 of the fecal DNA.

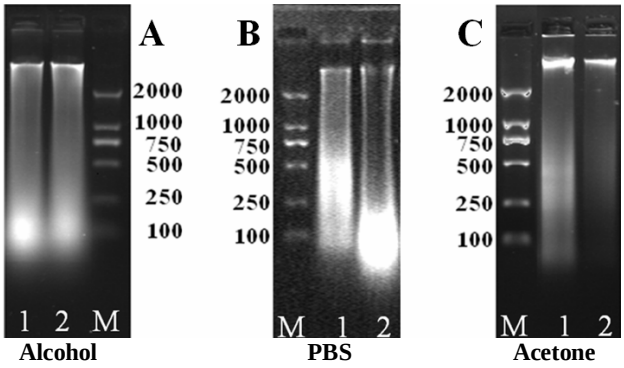

Fig. 1. The electrophoresis of faecal DNA distilled using different methods; A, alcohol extraction method; B, PBS extraction method; C, acetone extraction method; M, DL2000 markers; lanes 1 and 2, fecal DNA.

Amplificative quality from faecal DNA

The PCR products of D-loop and *Cyt b* with pygmy loris' fecal DNA extracted by different extract methods, the D-loop was about 400 bp (Fig.2A), and the *Cyt b* was near 1140 bp (Fig. 2B).

The amplification success rates of *Cyt b* with fecal DNA obtained by alcohol extraction method, PBS extraction method and acetone extraction method, were 20.98±0.83%, 22.94±0.72% and 32.51±0.55%, respectively. (Fig.3A). The amplification success rates of D-loop with fecal DNA obtained by alcohol extraction method, PBS extraction method and acetone extraction method were 20.47±0.81%, 25.17±0.53% and 40.06±0.62%, respectively (Fig.3B). The amplification success rates of *Cyt b* or D-loop with fecal DNA obtained by acetone extraction method was higher than that by alcohol extraction method and PBS extraction method. It is suggested that we can obtain high quality and quantity of fecal DNA by acetone extraction method.

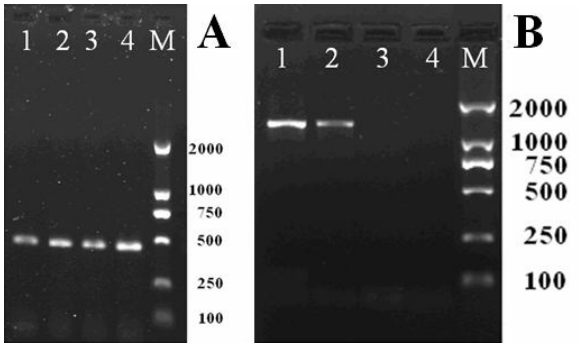

Fig. 2. Efficient amplification of pygmy loris different gene (A: D-loop gene; B: *Cyt b* gene; M, DL2000 markers; 1-4, amplified PCR products).

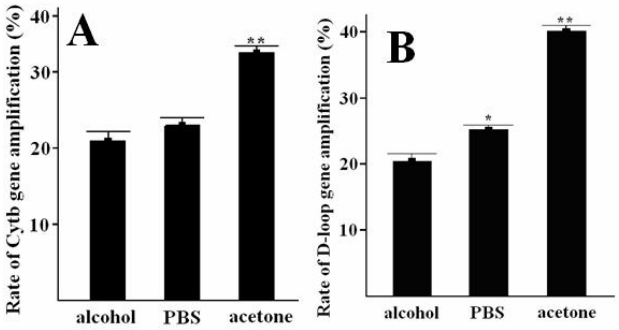

Fig. 3. The rate of amplification from the pygmy loris facal DNA.

DISCUSSION

A wide size range of host mitochondrial, microsatellite and even protein encoding nuclear DNA can be amplified from fecal extracts (Wasser *et al*., 1997). The feces are a plenty of resource for noninvasive sampling (Zhong *et al.*, 2003). The DNA extraction from feces can be used for genetic analysis of population, species and/or individual identification. It can also help the field ecological studies for determination of population estimates and delimiting of territory (Wang, 2001).

The component of feces is complex. It not only contains the animal intestinal cell DNA, but also DNA of parasites (bacteria, viruses) and the undigested food. The process of eluted feces by pro-

cool acetone with 2 or 3 times, will wipe off many PCR inhibitors (Schneiderbauer et al., 1991, Wang and Wang, 2012). We obtained an average of 23.14 μg/g of fecal DNA.

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