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An efficient protocol for genomic DNA extraction from the endangered species *Rhinopithecus brelichi*

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The Guizhou snub-nosed monkey (*Rhinopithecus brelichi*) is an endangered primate with approximately 750 individuals living in the Mount Fanjing area of northwestern Guizhou province. It is required to understand its evolutionary history and genetic diversity in order to take better measures for protecting this endangered species. As a widely accepted DNA source, faeces of snub-nosed monkeys were collected and used for DNA extraction. We modified the traditional method by washing the intestinal exfoliated cells with acetone and treated the cell lysate with high temperature (just below boiling point at 94 °C). We obtained a high-quality DNA, which was used for polymerase chain reactions (PCRs). Taking the mitochondrial genes (including ND6, tRNA-Glu and Cyto B region) of Sichuan snub-nosed monkey as reference, we performed a PCR and efficiently obtained DNA fragments with expected size. Further sequencing results showed that the amplified mitochondrial gene fragments had a high identity (percentage) with the published gene sequence of Sichuan snub-nosed monkey. These results demonstrated that this modified protocol is reliable and can be used in the genetic studies of monkeys and related species.

Keywords: *Rhinopithecus brelichi*; faecal DNA extraction; mitochondrial gene; PCR

Introduction

The Guizhou snub-nosed monkey (*Rhinopithecus brelichi*), which belongs to the order *Primates*, family *Cercopithecidae*, genus *Rhinopithecus*, is a unique and endangered primate in China. Approximately 750 individuals live in a small region in the Mount Fanjing area of northwest Guizhou province, over a range of 275 km² in Fanjing Mountain National Nature Reserve.[1,2] *R. brelichi* is categorized as an endangered species by the World Conservation Union and is also listed as a category I species under Chinese Wild Animal Protection Law in 1989. [1] Understanding its evolutionary history and current genetic structure will improve the conservation of its genetic diversity. It is highly desirable to get a high-quality DNA and to perform conservation and evolutionary studies in this species. However, the genetic study of *R. brelichi* individuals is limited due to difficulties in invasive sampling methods, where blood or tissue samples have to be obtained from these primates in the wild.[1,2] On the contrary, non-invasive samples, such as shed hair and faeces, are easier to collect and provide DNA samples without the need for capturing. Among these samples, faeces, which contain millions of intestinal exfoliated cells, have become the most potential DNA resources.[3,4] Höss et al. [5] used faecal genome DNA to analyse a 141 base

pairs (bp) fragment of mitochondrial DNA from brown bears (*Ursus arctos*), and initiated the technology of using a faecal DNA. Reed et al. [6] put forward a molecular scatology and launched individual and sex identification, population genetic variation, population size prediction, etc., based on polymerase chain reaction (PCR) of the faecal DNA samples. The reliability of faecal DNA extraction was confirmed by our group and others, in Sichuan snub-nosed monkeys (*Rhinopithecus roxellana*),[7] lions (*Puma concolor*),[8] baboons (*Papio cynocephalus ursinus*) and Asiatic black bears (*Ursus thibetanus*).[9,10] However, problems (i.e. PCR inhibitors in faeces and commercial DNA extraction kits being relatively expensive) still exist and hamper the use of previously published protocols for faecal DNA extraction. Therefore, there is a need for developing a simpler and cost-effective protocol for efficient faecal DNA extraction.

In the present study we reported a modified protocol by introducing two additional steps to our previous method,[10] which were washing the faecal cells with acetone and treating the cell lysate at 94 °C (just below boiling temperature). These two steps were adopted to remove PCR inhibitors and proteins. Using the resulting faecal DNA, bigger fragments of mitochondrial genes were successfully amplified by PCR. This study provides

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a cost-effective and relatively simpler method for faecal DNA extraction, which can be used in the genetic studies of monkeys and related species.

Materials and methods

Sample collection

Faecal samples were collected from the *R. brelichi* captive breeding farm in the Administration of Fanjing Mountain National Nature Reserve. Some hair samples were also collected and used for comparison. Faecal samples were stored in 100% ethanol at -20°C and hair samples were stored directly at -20°C .

Faecal DNA extraction

- (1) Add 20 mL of TE (10 mmol/L Tris-HCl 1 mmol/L EDTA (Ethylene Diamine Tetra Acetic acid), pH 8.0) into a sterilized beaker;
- (2) Transfer 2.5 g of faeces to the beaker, stir thoroughly and incubate for 2 min to allow samples to sediment;
- (3) Transfer 1 mL of the supernatant to 10 and 1.5 mL Eppendorf tubes.
- (4) Spin at 500 rpm for 5 min at 4°C ;
- (5) Transfer the supernatants into new Eppendorf tubes; spin at 1,500 rpm for 5 min at 4°C ;
- (6) Discard the supernatants and collect the pellets, which are crude intestinal exfoliated cells;
- (7) Wash the cell pellets with 0.5 mL pre-cooling acetone (0°C), spin at 1500 rpm for 5 min at 4°C .
- (8) Repeat step 7 two more times;
- (9) Pour off the acetone and dry the tubes;
- (10) Resuspend the pellets in 600 μL of Tris-EDTA (TE) buffer;
- (11) Add 40 μL of lysozyme (50 mg/mL) to the tube and incubate for 30 min at 37°C ;
- (12) Spin at 1500 rpm for 5 min at 4°C and discard the supernatant;
- (13) Add 500 μL of lysis buffer [200 mmol/L NaCl, 100 mmol/L Tris-HCl (pH 8.0), 2.0% SDS (Sodium Dodecyl Sulfate), 50 mmol/L EDTA, 1.0% Triton X00] and 12.5 μL Proteinase K (20 mg/mL) to each tube;
- (14) Incubate the samples for 3 hours or overnight at 55°C .
- (15) Add 1.25 μL of RNase (10 mg/mL) and incubate for 30 min at 37°C .
- (16) Incubate the tubes at 94°C for 10 min, spin at 13,200 rpm for 5 min at 4°C .
- (17) Add twice the volume of absolute ethanol to the lysate, keep at -20°C for 30 min or overnight;
- (18) Spin at 3200 rpm for 15 min at 4°C ;

- (19) Wash twice the pellet with 0.5 mL of ice-cold 70% ethanol;
- (20) Spin at 13,200 rpm for 5 min at 4°C ;
- (21) Dry the DNA pellets;
- (22) Resuspend the pellet in 50 μL of TE buffer and store at -20°C .

The hair DNA extraction was carried out according to the Higuchi's protocol.[11] To prevent cross contamination, DNA extraction of hair samples and faecal samples was carried out at different times in different labs.

PCR amplification of mitochondrial gene

According to the reported mitochondrial genome sequence of Sichuan snub-nosed monkey (*Rhinopithecus roxellana*) on NCBI (National Center for Biotechnology Information, GenBank No. JQ821835), the primers were designed as follows: Forward primer (the initiation site was 14042) 5'-TAG GAG AAG GCT TAG AAG AG', Reverse primer (the end site was 14738) 5'-AAG TGG AGG GTG AAG AAT C' (Generay Biotechnology Company, Shanghai). We also designed PCR primers to amplify the promoter region of the beta-actin gene by using the genome sequence of the rhesus macaque (*Macaca mulatta*) (Rhesus monkey, University of South Carolina [UCSC] Genome Browser assembly ID: rhe-Mac3). The used primers were: Forward primer 5'-CCC TGG AGA AGA GCT ACG AG', Reverse primer 5'-AGC CAT GCC CAA TCT CAT CT'. Amplification was performed in a total volume of 30 μL , containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl_2 , 0.1 mmol/L of dNTP, 0.4 $\mu\text{mol/L}$ of each primer, 1.5 U *Taq* DNA polymerase and 100 ng of genomic DNA. Thirty-six cycles were run on a Bio-Rad PTC00 thermocycler with pre-denaturing at 95°C for 6 min, denaturing at 94°C for 60 s, annealing at 51°C for 40 s, primer extension at 72°C for 80 s and final 8 min of extension at 72°C . Negative (water) controls were used to check PCR performance and potential contamination.

Electrophoresis and sequencing

The results of the DNA extraction and the PCR amplifications were analysed by gel electrophoresis, on a 0.8% and 1.5% agarose gel, respectively. PCR products were sequenced on ABI 377 Genetic Analyzer. To avoid errors, PCR amplification and sequencing were performed in triplicate.

DNA sequence alignment

DNA alignment of mitochondrial gene (ND6, tRNA-Glu and Cyto B genes) was performed by DNAMAN5.29 with several primates, such as *Rhinopithecus avunculus*

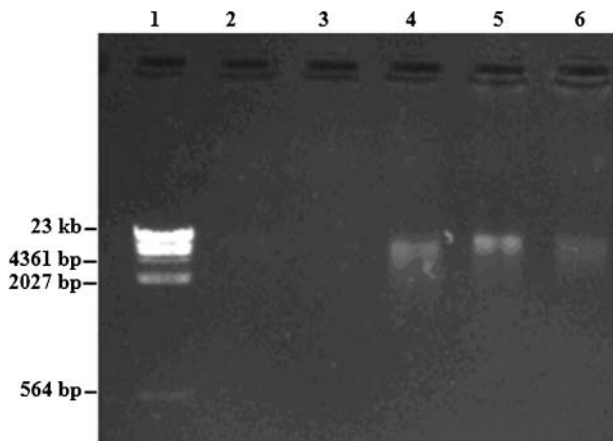


Figure 1. Gel electrophoresis pattern of faecal and hair genomic DNA from *R. brelichi*.
Note: *Hind* III digested λ DNA Marker (1); negative control of extraction (2); hair DNA sample (3); faecal DNA samples (4–6).

(JF293093), *Pygathrix roxellana* (DQ355300), *Rhinopithecus roxellana* (JQ821835), *Rhinopithecus bieti* (JQ821839), *Nasalis concolor* (JF293095) and *Rhinopithecus strykeri* (JQ821838), because of their close relationship. The published *R. brelichi* (JN540032) was also listed in for testing quality of genomic DNA from faeces.

Results and discussion

Successful extraction and amplification of faecal DNA from *R. brelichi*

For the performance of genetic studies on endangered species, such as Guizhou snub-nosed monkey (*Rhinopithecus brelichi*), obtaining genomic DNA is critical. Conventionally, even for common species, blood or tissue samples are difficult to obtain. Therefore, faeces would be the ideal

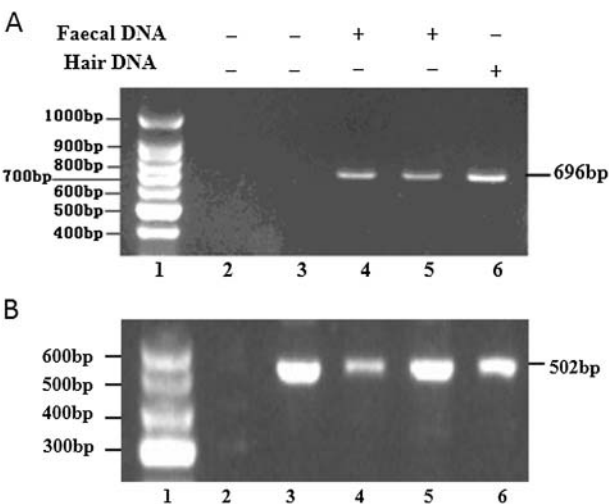


Figure 2. PCR amplification of *R. brelichi* mitochondrial genes.
Note: PCR amplification of *R. brelichi* mitochondrial genes (A); DNA Marker (1); negative control of amplification (2); negative control of extraction (3); faecal DNA samples (4, 5); hair DNA sample (6); PCR amplification of *R. brelichi* genes (B); marker (1); negative control of amplification (2); faecal DNA samples (3–6).

experimental material because it is taken using a non-invasive sampling method and it is the most simple and informative material.[12] Albaugh et al. [13] demonstrated that 3.0×10^6 to 4.0×10^6 intestinal exfoliated cells could be obtained from 1 g of fresh stools and most of them showed viability. Taberlet et al. [14] reported that the multiple-tubes approach could obtain reliable genotypes with a confidence level of 99%, when using faecal samples.

Here we referred to the protocol reported by Zhang et al. [10] and used an improved method for faecal DNA

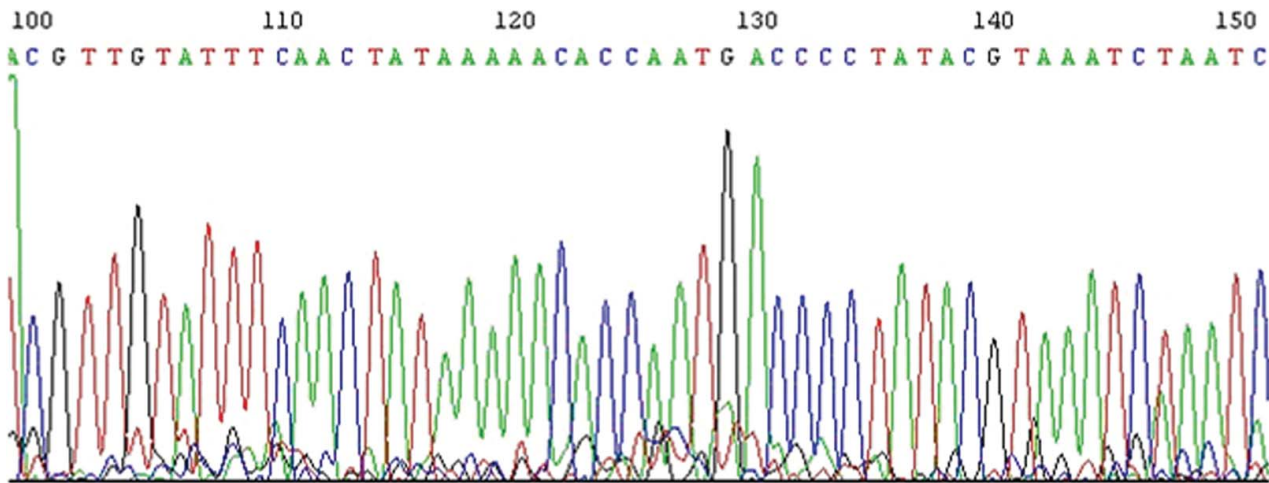


Figure 3. Partial sequencing result of *R. brelichi*'s mitochondrial gene.
Note: This is a partial sequencing result from *R. brelichi* mitochondrial gene, showing the sequencing quality.

<u>R. brelichi</u>	CGTTGTATTTCAACTATAAAAAACACCAATGACCCCTATACGTAAATCTAA	50
<i>Rhinopithecus avunculus</i>	-----g-----t-----	50
<i>Pygathrix roxellana</i>	-----g-----	50
<i>Rhinopithecus roxellana</i>	-----g-----	50
<i>Rhinopithecus bieti</i>	-----a-----a-----a-----g-----a-----	50
<i>Nasalis concolor</i>	-----a-----	50
<i>Rhinopithecus strykeri</i>	-----g-----	50
<i>Rhinopithecus brelichi</i>	-----g-----	50
<u>R. brelichi</u>	TCTAATTATAAAAAATAATTAATCACTCTCTTATTGATCTACCCACCCCAT	100
<i>Rhinopithecus avunculus</i>	-----a-----t-----t-----	100
<i>Pygathrix roxellana</i>	-----a-----t-----	100
<i>Rhinopithecus roxellana</i>	-----a-----t-----	100
<i>Rhinopithecus bieti</i>	-----c-----g-----g-----t-----	100
<i>Nasalis concolor</i>	-----t-----t-----c-----g-----t-----t-----	100
<i>Rhinopithecus strykeri</i>	-----c-----	100
<i>Rhinopithecus brelichi</i>	-----c-----t-----g-----	100
<u>R. brelichi</u>	CAAAATATTTTCAGCATGATGAAACTTCGGTTCCTTCTAGCAACTTCTTTA	150
<i>Rhinopithecus avunculus</i>	-----c-----a-----t-----g-----c-----	150
<i>Pygathrix roxellana</i>	-----t-----t-----	150
<i>Rhinopithecus roxellana</i>	-----t-----t-----	150
<i>Rhinopithecus bieti</i>	-----c-----c-----a-----t-----	150
<i>Nasalis concolor</i>	-----c-----ca-----c-----a-----	150
<i>Rhinopithecus strykeri</i>	-----c-----c-----t-----	150
<i>Rhinopithecus brelichi</i>	-----c-----t-----	150
<u>R. brelichi</u>	ATATTACAAATTATTACTGGCTTATTTCTAGCAATACACTACTCACCAGA	200
<i>Rhinopithecus avunculus</i>	-----a-----a-----	200
<i>Pygathrix roxellana</i>	-----t-----t-----	200
<i>Rhinopithecus roxellana</i>	-----t-----t-----	200
<i>Rhinopithecus bieti</i>	-----c-----c-----	200
<i>Nasalis concolor</i>	-----t-----g-----c-----g-----c-----t-----	200
<i>Rhinopithecus strykeri</i>	-----t-----	200
<i>Rhinopithecus brelichi</i>	-----t-----	200
<u>R. brelichi</u>	CACCTCCTCCGCCTTCTCCTCAATCGCCCATATCACCCGAGACGTAAACT	250
<i>Rhinopithecus avunculus</i>	-----t-----t-----	250
<i>Pygathrix roxellana</i>	-----t-----t-----	250
<i>Rhinopithecus roxellana</i>	-----t-----t-----	250
<i>Rhinopithecus bieti</i>	-----t-----t-----g-----c-----	250
<i>Nasalis concolor</i>	-----t-----t-----t-----t-----c-----	250
<i>Rhinopithecus strykeri</i>	-----t-----t-----c-----	250
<i>Rhinopithecus brelichi</i>	-----t-----t-----	250
<u>R. brelichi</u>	ATGGTTGAACATATCCGCTACCTCCACGCTAATGGCGCCTCTATATTCTTT	300
<i>Rhinopithecus avunculus</i>	-----c-----c-----c-----t-----	300
<i>Pygathrix roxellana</i>	-----c-----c-----t-----	300
<i>Rhinopithecus roxellana</i>	-----c-----c-----t-----	300
<i>Rhinopithecus bieti</i>	-----c-----t-----t-----c-----t-----c-----	300
<i>Nasalis concolor</i>	-----c-----t-----t-----t-----c-----t-----c-----	300
<i>Rhinopithecus strykeri</i>	-----t-----t-----t-----c-----t-----	300
<i>Rhinopithecus brelichi</i>	-----t-----t-----	300
<u>R. brelichi</u>	ATCTGCCTATTCTTACACGTAGGTCGAGCATATATATTATGGCTCATTCTCT	350
<i>Rhinopithecus avunculus</i>	-----g-----g-----c-----	350
<i>Pygathrix roxellana</i>	-----g-----g-----c-----	350
<i>Rhinopithecus roxellana</i>	-----t-----t-----c-----	350
<i>Rhinopithecus bieti</i>	-----t-----t-----c-----	350
<i>Nasalis concolor</i>	-----t-----c-----c-----c-----	350
<i>Rhinopithecus strykeri</i>	-----t-----c-----c-----	350
<i>Rhinopithecus brelichi</i>	-----t-----c-----	350
<u>R. brelichi</u>	TCTTATTGAAACCTGAAACATTGGCATTGCACTACTATTTCATAAACCATGG	400
<i>Rhinopithecus avunculus</i>	-----g-----t-----t-----c-----t-----a-----	400
<i>Pygathrix roxellana</i>	-----g-----t-----t-----c-----t-----a-----	400
<i>Rhinopithecus roxellana</i>	-----g-----t-----t-----c-----t-----a-----	400
<i>Rhinopithecus bieti</i>	-----c-----cc-----t-----t-----ca-----ct-----c-----t-----a-----	400
<i>Nasalis concolor</i>	-----c-----cc-----t-----t-----ca-----ct-----c-----t-----a-----	400
<i>Rhinopithecus strykeri</i>	-----t-----t-----t-----c-----	400
<i>Rhinopithecus brelichi</i>	-----t-----t-----t-----c-----	400
<u>R. brelichi</u>	CAACAGCCTTTATAGCCTACGTACTCCCATGAGGACAAATATCATCTCTCA	450
<i>Rhinopithecus avunculus</i>	-----g-----g-----	450
<i>Pygathrix roxellana</i>	-----g-----g-----	450
<i>Rhinopithecus roxellana</i>	-----g-----g-----	450
<i>Rhinopithecus bieti</i>	-----c-----t-----t-----t-----t-----	450
<i>Nasalis concolor</i>	-----c-----t-----t-----t-----t-----	450
<i>Rhinopithecus strykeri</i>	-----t-----t-----	450
<i>Rhinopithecus brelichi</i>	-----t-----t-----	450
<u>R. brelichi</u>	GCTGCCACAGTAATTACAAACCTTACTATCGGCAATTCATATATCGGAAC	500
<i>Rhinopithecus avunculus</i>	-----t-----c-----g-----	500
<i>Pygathrix roxellana</i>	-----t-----c-----g-----	500
<i>Rhinopithecus roxellana</i>	-----c-----t-----t-----t-----c-----t-----g-----	500
<i>Rhinopithecus bieti</i>	-----c-----t-----t-----t-----c-----t-----g-----	500
<i>Nasalis concolor</i>	-----c-----t-----t-----t-----c-----t-----g-----	500
<i>Rhinopithecus strykeri</i>	-----c-----t-----t-----t-----c-----t-----g-----	500
<i>Rhinopithecus brelichi</i>	-----c-----t-----t-----t-----c-----t-----g-----	500
<u>R. brelichi</u>	TAATATTGTTTCAGTGCAGTTTGAGCTGGCTATTCCATTGATAACCCAACAC	550
<i>Rhinopithecus avunculus</i>	-----a-----a-----g-----g-----a-----t-----	550
<i>Pygathrix roxellana</i>	-----g-----a-----g-----g-----g-----	550
<i>Rhinopithecus roxellana</i>	-----g-----a-----g-----g-----g-----	550
<i>Rhinopithecus bieti</i>	-----c-----c-----a-----g-----a-----c-----t-----g-----t-----	550
<i>Nasalis concolor</i>	-----c-----c-----a-----g-----a-----c-----t-----g-----t-----	550
<i>Rhinopithecus strykeri</i>	-----g-----g-----	550
<i>Rhinopithecus brelichi</i>	-----g-----g-----	550
<u>R. brelichi</u>	TTACACGATTCTTCACCGCTCCAC	573
<i>Rhinopithecus avunculus</i>	-----	573
<i>Pygathrix roxellana</i>	-----	573
<i>Rhinopithecus roxellana</i>	-----	573
<i>Rhinopithecus bieti</i>	-----	573
<i>Nasalis concolor</i>	-----t-----t-----t-----	573
<i>Rhinopithecus strykeri</i>	-----	573
<i>Rhinopithecus brelichi</i>	-----t-----	573

Figure 4. DNA sequence alignment of mitochondrial genes (ND6, tRNA-Glu and Cyto B genes).

Note: The sequence of mitochondrial genes (including ND6, tRNA-Glu and Cyto B) of *R. brelichi* (underlined) was aligned with those from other closely related species. Dash line indicates the same base, whereas lowercase letter shows a different base at that position. These sequences can be retrieved from NCBI.

extraction. Instead of phenol-chloroform, we included a boiling method (94 °C, 10 min), which is used to denature and remove proteins in order to prepare the plasmid DNA. [15,16] We managed to obtain a high-quality genomic DNA (average concentration – 41.54 ng/μL, OD 260/280 = 2.18 (the ratio of optical density at wavelength of 260 and 280 nm)). Faecal genomic DNA with size of about 23 kb was detected through electrophoresis on a 0.8% agarose gel (Figure 1). The protocol that we modified made the faecal genome DNA extraction easier and effective, without the need of commercially available DNA extraction kits.

We also observed that we obtained a higher quality genomic DNA from faecal samples than the one obtained from hair samples, and the extraction process had no contamination. PCR products of the mitochondrial genome were detected on a 1.5% agarose gel (Figure 2(A)). As expected, specific 696 bp products were obtained repeatedly in multiple experiments. Consistently, products of the same size were also observed from the hair samples.

We included negative (water) and positive (hair) controls for faecal DNA extraction and PCR to avoid contamination. On average, seven out of nine tubes of the faecal samples gave positive results. Each faecal sample was used for DNA extraction and PCR amplification three times and all gave consistent results.

It has been shown that it is difficult to amplify DNA fragments with more than 600 bp from faecal genomic DNA, because of severe degradation [17]; however, we amplified a 696 bp mitochondrial gene fragment with our improved protocol. Furthermore, we successfully amplified a 502 bp DNA fragment of the beta-actin promoter region (Figure 2(B)). This fragment has also been confirmed by Sanger sequencing (data not shown). Thus, we were able to obtain much longer mitochondrial DNA fragments with our extraction method.

Sequencing results of the mitochondrial gene of *R. brelichi*

Sequence of mitochondrial fragment spanning ND6, tRNA-Glu and Cyto B genes (14,042–14,738 bp) was obtained and a partial sequencing result is shown in Figure 3. Currently, due to the lack of blood samples, genetic studies on *R. brelichi* are few and most reports are focused on the morphology, behaviour, ecological environment and artificial feeding.[18–21] Alignment result of mitochondrial gene for *R. brelichi* from our study with others is shown in Figure 4. We found that *R. brelichi* had the highest identity with *Rhinopithecus avunculus* (95.11%) and *Rhinopithecus roxellana* (95.11%) (Figure 5). The result indicated that *R. brelichi* has the nearest relationship with them. We also compared the sequence from our paper with the published *R. brelichi* sequence and obtained 99.65% identity. The difference might be due to individual genetic variations.

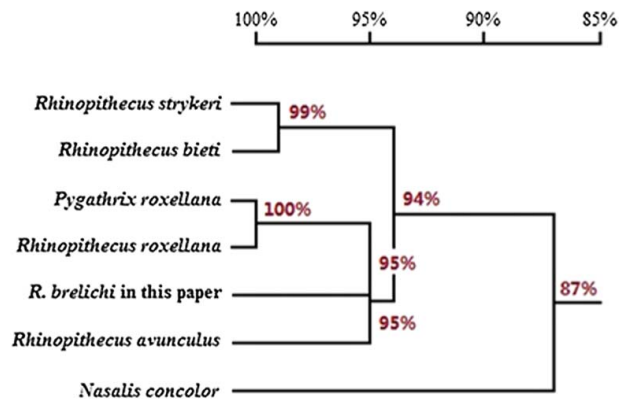


Figure 5. Homology tree from DNA sequencing of mitochondrial genes.

Note: This homology tree was generated with DNAMAN12.0 program. Based on the partial sequence of the mitochondrial genes that we sequenced, we observed the closest relationship between *R. brelichi*, *Pygathrix roxellana*, *Rhinopithecus roxellana* and *Rhinopithecus avunculus* (at least 95% identity for this region). The relationship between *R. brelichi* and *Nasalis concolor* was less—only 87% of the sequences were identical.

Conclusions

In the present study we provided an efficient protocol for genomic DNA extraction from the endangered species *Rhinopithecus brelichi*. We showed that faeces of wild animals, such as *R. brelichi*, can be used as an abundant resource of DNA extraction for genetic studies. Our modified protocol for faecal DNA extraction is simpler and more cost-effective, and is especially suitable for such studies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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