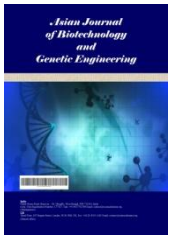

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A Simple and Effective Phenol-Chloroform Method of DNA Extraction from Mammalian Feces

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Authors' contributions

Author AN designed the protocol and the study, performed specimen collection, molecular analysis, data interpretation and manuscript writing, Author KS designed the study, performed specimen collection, molecular analysis and manuscript writing. Authors KR and PA performed supervision and Author DJ managed the substantiative manuscript review. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: To non-invasively collect the fecal samples of 12 different mammals and devise a simple method of fecal DNA extraction using a modified phenol-chloroform procedure of DNA isolation for species identification.
Study Design: The experiment was laid out to check the applicability of the devised protocol. Morphological identification was done in the field to collect samples from the wild. Molecular characterization was carried out in the Molecular laboratory for species identification.
Place and Duration of Study: Advanced Institute for Wildlife Conservation (AIWC), Tamil Nadu Forest Department, Vandalur, Chennai, Tamil Nadu. The samples were collected between the time period of September 2019 and March 2020. The molecular analysis was performed between July 2020 and July 2021.
Methodology: We devised a scat/ fecal DNA isolation protocol and tested its applicability on 81 samples (30 herbivores, 15 omnivores, 36 Carnivores). Fresh and old samples were collected from wild (n=41) and captive (n=40) areas and used for the study. Independent isolation for each species was carried out with extraction control. The DNA isolated samples were quantified for

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concentration using Nanodrop Spectrophotometer to calculate the optimum DNA concentration for amplification. Independent PCR amplification of mitochondrial regions of cytochrome b and 12S rRNA were performed and gel electrophoresis was carried out for each sample for positive amplicons. The PCR products were sequenced using genetic analyzer.

Results: The protocol was validated by checking the strength of the devised method to work on species belonging to different ecological types. The sample size is n=81, positive amplification in cytochrome b region is 71 and 12S rRNA region is 79. Success of devised DNA extraction protocol on different population kinds, such as wild (n=41) and captive (n=40) were evaluated with a ratio of 'Positive PCRs of samples' against 'Total samples for PCR'. It is 97.56 % (cytochrome b) and 100 % (12S rRNA) of Wild population and 60.0 % (cytochrome b) and 95% (12S rRNA) of captive population.

Conclusion: The devised protocol successfully worked on both wild and captive populations of herbivores, omnivores and carnivores. The success rate is better in 12S rRNA region on comparison with Cytochrome b. The applicability and reliability of the protocol has been tested and validated by checking the obtained sequences in the NCBI database and submitting the same to the database.

Keywords: Fecal DNA; mammals; molecular scatology; modified phenol-chloroform method.

1. INTRODUCTION

Wildlife being a massive expanse needs persistent backing of growing molecular technology to explore, study and unravel the atypical or abstract species, particularly for species identification which is fundamental for ecological studies, conservation, and associated management applications. Constantly advancing technology recognized non-invasive sampling as an easily accessible and safest sampling choice, concerning the scale of disturbance created to the animals by invasive sampling. The choice of feces as a specimen of study facilitates the clear indication of species' presence [1], preferable and beneficial over any other sample type.

In view of that, molecular scatology, the self-explanatory terms specifying the study of genetic material extracted from the fecal sample, has gained attention and interest globally since the recent past. Various laboratory protocols have been formulated for fecal DNA extraction such as guanidium thiocyanate silica method, phenol-chloroform method [2], glass-milk method [3]. The applications of these methods have limitations due to the presence of inhibitors, rate of sample degradation, sample condition and fundamentally the protocol complexity demanding large dollops of feces, several chemical components and longer extraction procedures. To overcome the above limitations commercial kit method of extraction is widely adapted, which is efficient, nevertheless expensive. Though commercial kits have reserved its chief utilization in regard to fecal DNA extraction, two major drawbacks of using any prescribed method are the non-applicability on herbivorous fecal material due to coloration, and low retention, recovery of both quality and quantity of DNA. Although fresh scats provide sufficient amounts of DNA for analysis, largely the study design involves longer sampling over a few weeks. For these reasons, an improved method of storage minimizing the degradation of DNA is highly crucial for sample viability and future analysis.

A convenient and exercisable approach for long-term storage coupled with considerable fecal DNA recovery from a wide range of taxa and ecological types would be exceptionally valuable, predominantly to the research groups working on population genetics of elusive species. This motivated to devise and propose a new method of fecal DNA extraction laboratory protocol to find application and pliancy with routine laboratory consumables and tailored to a wide range of mammalian species. The list of mammalian species picked for this study were based on their ecological importance and illegal trade value and protection status (The Wildlife Protection Act, 1972). The study also displays the classification of selected species on its dietary nature such as herbivores, carnivores and omnivores to prove efficacy on DNA retrieval from animal fecal material.

2. MATERIALS AND METHODS

2.1 Sample Collection

The samples were collected from free ranging and captive mammalian species of Tamil Nadu

Forest Department between September 2019 and March 2020. The Samples were transported to the laboratory within 7-10 days and stored in a dry place for extraction.

2.2 Sample Preservation

All samples were collected in clean air-tight containers containing silica gel beads filled to their half, wherein the sample and the gel beads were separated by paper towel, so as to absorb the moisture that may result in undesirable microbial growth [4]. Post collection the samples were dried under the warmth of sunlight, devoid of direct exposure. Such a method of preservation allows long-term preservation and storage of scat irrespective of the diet habits of the mammalian species.

2.3 DNA Extraction

DNA extraction was carried out through swabbing using Longmire buffer (100mM Tris 100mM EDTA, 10mM NaCl, 0.5% SDS, Autoclaved water, pH 8.0) [5], to enable the retention of epithelial cells rich in superficial layer. Centrifuge tubes of 2mL containing 850 µL Longmire buffer with sterile sample swab (Himedia), 25 µL of 20 mg/mL proteinase K (KAPA Biosystems, SIGMA) and 25 µL of 10mM of DTT were added and samples were subjected to incubation at 56 °C for 4 hrs. A modified phenol chloroform method of extraction was followed on completion of digestion process [6] with decreased chemical/ reagent reaction time to 1 min of inversion mixing. The samples were incubated at (-20°C) overnight, for precipitation of DNA using 1 volume of (96 - 100%) Isopropyl Alcohol. The resulting pellet was washed with 70 % ethanol twice, air-dried and eluted in 30-60 µL of autoclaved nuclease free water depending on the size, transparency and coloration of the pellet. The samples of each species were isolated independently with an extraction control to monitor for contamination.

The listed species are based on the classification of their feeding routine. PCR amplification of mtDNA markers Cytochrome b with L14841 & H15141 and 12S rRNA with L1091 & H1478, L1085 & H1259 markers were carried out. The asterisked numbers denote complete successful amplifications.

2.4 PCR and Sequencing

The partial fragments of mitochondrial DNA such as Cytochrome b [7] yielding 370bp, 12S rRNA [8] yielding 215bp, and 12S rRNA [7] yielding 500bp were amplified. The PCR amplification was carried out in Eppendorf Nexus GSX1 Mastercycler in 10 µL reaction volumes. Each PCR reaction was prepared comprising 1X Taq Buffer (KAPA Biosystems, SIGMA), 0.25 mM dNTPs, 0.4 µM of both forward and reverse primer, 2.5 mM MgCl2, 0.25 U Taq DNA Polymerase (KAPA Biosystems, SIGMA) and 1 µL of template DNA. Cycling conditions consisted of 5 min. of initial denaturation at 95 ˚C, followed by 35 cycles of 30 seconds of denaturation at 95 ˚C, 30 seconds of annealing at 55 ˚C (Cytochrome b, 12S) [7] and 57˚C (12S)

Table 1. List of species sampled

Species Name	Sample Number	Positive amplification	
		Cytochrome b	12S rRNA
HERBIVORES			
Elephant (*Elephas maximus*)	23	23*	23*
Indian gaur (*Bos gaurus*)	2	1	1
Sambar deer (*Rusa unicolor*)	3	3*	3*
Black Naped Hare (*Lepus nigricollis*)	2	2*	2*
OMNIVORES			
Wild pig (*Sus scrofa*)	2	2*	2*
Sloth bear (*Melursus ursinus*)	4	2	4*
Slender loris (*Loris lydekkerianus*)	2	2*	2*
Jackal (*Canis aureus*)	7	4	6
CARNIVORES			
Tiger (*Panthera tigris*)	13	11	13*
Wild dog - Dhole (*Cuon alpinus*)	11	10	11*
Mongoose (*Herpestes edwardsii*)	5	5*	5*
Hyaena (*Hyaena hyaena*)	7	6	7*
TOTAL	**81**	**71**	**79**

** indicates that positive amplification success per sample using each marker is the same as sample number*

[8], 45 seconds of extension at 72 ˚C and final extension at 72˚C for 10 min. The 12S PCR [8] was set up with primer concentration of 0.5 µM of both forward and reverse primer. The PCR reactions were set with positive and non-template controls. The PCR amplicons were visualized in 2% agarose gel with novel juice stain (SIGMA) and documented using BioRad XR+ gel doc system. The PCR samples were purified using QIAquick gel extraction kit (Qiagen, Germany) and sequences were obtained through Sanger sequencing performed in ABI 3730 Genetic Analyzer (Applied Biosystems, USA). The forward and reverse sequences were trimmed at both the ends using MEGA X software [9]. The authenticity of the sequenced samples was confirmed by match against the NCBI database and through submission of sequences to the NCBI database. During PCR experiments each sample lot was monitored for contamination by inclusion of PCR positive control, DNA extraction control, PCR No-template control (No-DNA). To confirm the reliability of the DNA extraction protocol, the products were checked for positive amplification in no sample extraction control.

3. RESULTS AND DISCUSSION

By and large, the assessment of any novel protocol for fecal DNA extraction is challenging due to a few key reasons such as: 1. The DNA quantity from the animal feces is less, 2. High possibility of unintended microbial DNA extraction [10,11], 3. Fragmented target gene regions. Hence, quantification of DNA from fecal samples is unreliable. We recommend quantification only for the purpose of finding the optimal DNA concentration for a workable PCR reaction.

According to our experimental design, the protocol was followed with default 4 hours of extraction digestion time, reduction reflects in the DNA concentration. The reduction in reaction time of each chemical treatment in the phenol-chloroform procedure resulted in isolation of intact DNA of the sample as displayed in the gel picture (Fig. 1) where large amounts of DNA extracted with higher band intensity clearly attributes to the strength of the devised protocol.

3.1 DNA Extracted Fecal Samples

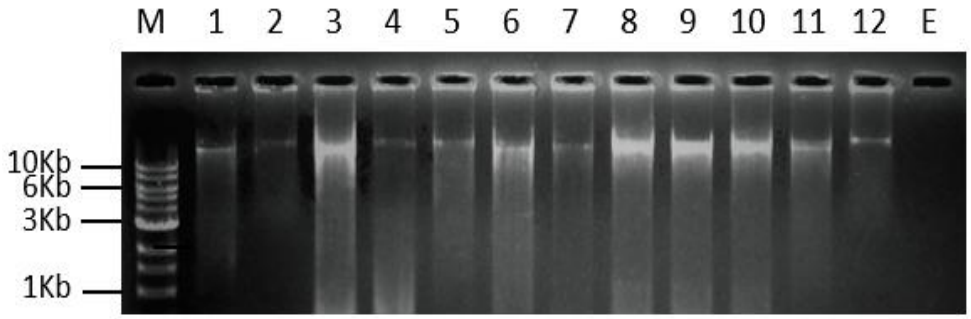
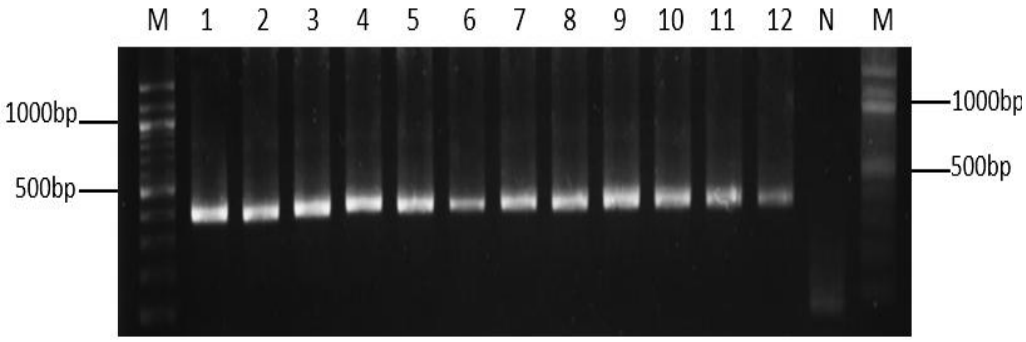


Fig. 1. Fecal DNA from 12 Tiger (*Panthera tigris*) samples detected on a 0.8% agarose gel stained with novel juice stain. Lane M- DNA ladder, Lane E – Extraction control

3.2 Gel Result of PCR Amplified Samples Cytochrome b and 12S rRNA Products



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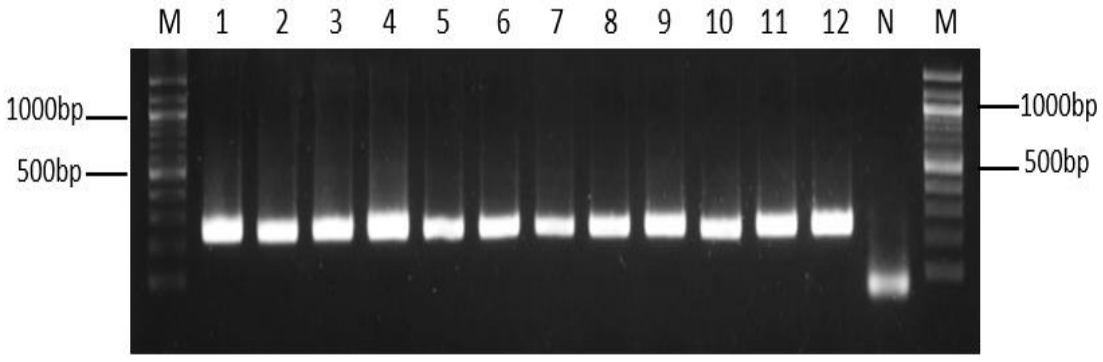

Fig. 2 (A, B). Amplified products of mtDNA cytochrome b gene (A) of 370bp from fecal DNA of different mammals using primers L14841and H15149 and 12S rRNA gene (B) from fecal DNA of different mammals using primers L1085 and H1259 yielding the size range of 215bp to 240bp, detected on a 2.0% agarose gel, the products were stained with Novel Juice Stain (NJS). Lanes: 1, Indian Elephant; 2, Gaur or Indian Bison; 3, Sambar deer; 4, Black Naped Hare; 5, Wild pig; 6, Sloth bear; 7, Slender loris; 8, Jackal; 9, Tiger; 10, Wild dog or Dhole; 11, Mongoose; 12, Hyaena; M – 100bp DNA ladder; N – Negative control

Further, validity of the devised protocol was observed in successful amplifications in almost all the different species using polymerase chain reaction. The gel images (Figs. 2 & 3) of cytochrome b and 12S rRNA markers' amplification display the integral conservation of cytochrome b and 12S rRNA mtDNA regions. To check the amplification ability in obtaining amplicons of higher size range of 450bp to 550bp, we used primer set L1091/H1478 on a few species with a positive control (Fig. 3).

The average percentage (Table 2) of positive amplification on either of the population kinds with primer set L1085/H1259 is between 97.5 to 100%, and that of primer set L14841/ H15149 is 78.88 %. On a performance comparison, 12S rRNA markers showed better amplification to Cytochrome b marker. All mitochondrial regions employed in this study allowed appropriate identification of all classes of species, the Herbivores, Carnivores and Omnivores, tested using scat samples.

The local alignment BlastN tool was used for identification of species, which revealed known species match with the expected species. A few partial sequences of both the mtDNA markers were submitted to the NCBI database for each species. The BankIt submissions of cytochrome b and GenBank submissions of 12S rRNA are listed in the table (Table 3). All the listed species were successful in both the primers, except for Indian gaur, Sloth bear, Jackal in sequencing of cytochrome b region. This would be due to fragmented cytochrome b region in the DNA isolated from their fecal samples.

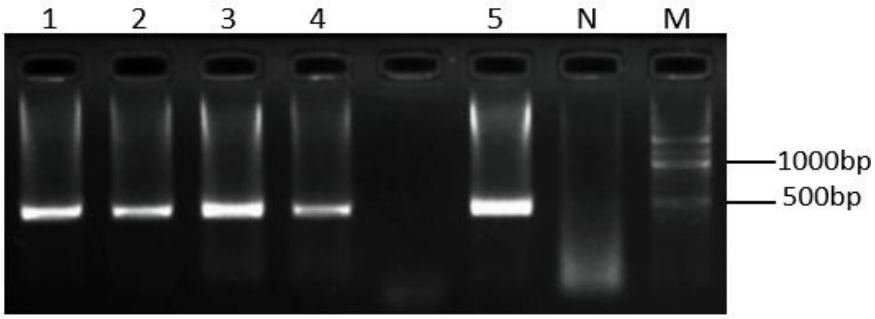

Fig. 3. Amplified products of mtDNA 12S rRNA gene from fecal DNA of different mammals using primers L1091 and H1478 yielding the size range of 450bp to 500bp, detected on a 2.0% agarose gel, the products were stained with Novel Juice Stain (NJS). Lanes: 1, Wild pig; 2, Slender loris; 3, Tiger; 4, Hyaena; 5, Positive Control; M – 100bp DNA ladder; N – Negative control

Table 2. Success of devised DNA extraction protocol on different population

Population Kind	Sample Number	Successful amplification (Positive PCRs of samples/ Total samples for PCR)	
		Cytochrome b	12S rRNA
Wild	41	97.56 % (40/41)	100 % (41/41)
Captive	40	60.0 % (24/40)	95 % (38/40)

Table 3. Submissions to the NCBI database

Species	Accession number	
	Cytochrome b	12S rRNA
Elephant (*Elephas maximus*)	MW999345	MW900440
Indian gaur (*Bos gaurus*)	-	MZ427324
Samber deer (*Rusa unicolor*)	MZ436174	MW979410
Black Naped Hare (*Lepus nigricollis*)	MW999344	OM022006
Wild pig (*Sus scrofa*)	MZ540014	MZ531907
Sloth bear (*Melursus ursinus*)	-	MZ427323
Slender loris (*Loris lydekkerianus*)	MW924861	MW899376
Jackal (*Canis aureus*)	-	MZ405567
Tiger (*Panthera tigris*)	MZ927339	MZ408531
Wild dog - Dhole (*Cuon alpinus*)	MW911472	MW904022
Mongoose (*Herpestes edwardsii*)	MZ540015	OK103788
Hyaena (*Hyaena hyaena*)	MZ331839	MZ088142

DNA quantification showed that our modified method yielded more than adequate concentration in most of the samples subjected to experiment (Table 4). Likewise, it is commonly shown that a low amount of template DNA is inappropriate for successful amplification of target gene region [3], contradictorily, we used concentrations as low as 5ng/µL and achieved successful amplifications.

The successful amplification was partly due to the modified preservation method [4] which is simple to follow, yet effective for quite a long duration, up to two years without the need for any sophisticated chemical treatment compared to the previous fecal DNA isolation methods [2,11,12,13,14,15]. The experiment was designed to assess the applicability of the preservation and extraction protocol in mammalian scat samples of different dietary intake. This is because the feces of herbivorous animals contain polysaccharides and phenolic substance from plant tissue [13] while carnivorous animal feces contain bile salts and bilirubin [12], all these acts as a strong inhibitor of DNA utilization during isolation and PCR process. Previous studies on modified phenol-chloroform methods [12 & 15] applied either chemical treatment or freezing for preservation of samples.

Table 4. Quantification of concentration of fecal DNA samples

Species name	Sample count	CONC (ng/ µL) (Range)
Elephant (*Elephas maximus*)	23	84 – 188
Indian gaur (*Bos gaurus*)	2	15 – 27
Samber deer (*Rusa unicolor*)	3	70 – 96
Black Naped Hare (*Lepus nigricollis*)	2	22 – 29
Wild pig (*Sus scrofa*)	2	210 - 456
Sloth bear (*Melursus ursinus*)	4	35 - 42
Slender loris (*Loris lydekkerianus*)	2	15 - 33
Jackal (*Canis aureus*)	7	23.8 - 30
Tiger (*Panthera tigris*)	13	56 - 318.3
Wild dog - Dhole (*Cuon alpinus*)	11	65 - 306
Mongoose (*Herpestes edwardsii*)	5	25 - 47
Hyaena (*Hyaena hyaena*)	7	450 - 870

But the successful elimination of potent PCR barriers without inclusion of any such treatments shows the higher efficiency of our method to existing conventional fecal DNA extraction methods.

4. CONCLUSION

The modified phenol chloroform method for DNA isolation from mammalian faeces has proven to be an inexpensive alternative when it comes to unassured results of expensive commercial kits. Therefore, this method is best suitable to ascertain the species identity of mammals in the wild where only faeces is the available source of biological sample. This method proved to be a convenient and an exercisable approach for long-term storage coupled with considerable fecal DNA recovery from a wide range of taxa and ecological types. Hence, this work would be exceptionally valuable to the research groups working on phylogeography, genetics and population ecology of wildlife species relying on fecal samples as study specimens.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. This research was funded by the core funds of Advanced Institute for Wildlife Conservation (AIWC), Tamil Nadu Forest Department (TNFD), Tamil Nadu.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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