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NON-INVASIVE DNA EXTRACTION FOR MOLECULAR IDENTIFICATION OF ROYAL BENGAL TIGER *PANTHERA TIGRIS TIGRIS*

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Abstract

The flagship animal species of Sundarbans, the Royal Bengal tiger (*Panthera tigris tigris)* is under threat of extinction. Its natural population is declining day by day. So, to avoid killing and harming the animal, the use of non-invasive samples such as scat, hair, or scent is preferred for DNA extraction and subsequent genotyping of tiger species. DNA has been extracted from scat samples of the Bengal tiger in the present study, and a fragment of the *cytochrome b* gene has been sequenced after PCR with species-specific primers. DNA has been extracted manually using a previously described methodology with slight modifications. The size of the PCR product and sequence of *cytochrome b* gene indicates that tiger DNA is successfully extracted from scat samples using tiger-specific primers. Thus, presence of tiger DNA can be detected by using this method just by the PCR product size in the gel. This is the first report of a partial sequence of mitochondrial *cytochrome b* gene of *P. t. tigris* from Bangladesh.

Introduction

Bengal tiger, scientifically known as *Panthera tigris tigris* (Carnivora : Felidae), is the largest and most attractive cat species of Sundarbans(1). Tiger is at the tertiary trophic level of the ecological pyramid of the mangrove forest. It prefers to prey on deer, boars, mammals, etc. Since it plays a pivotal role in protecting overall biodiversity, it is recognized as the umbrella or flagship species of Sundarbans(2). However, due to habitat degradation and fragmentation, illegal killing, and capture, its number decreases day by day. As a consequence, the status of the Bengal Tiger (*P. t. tigris*) has been endangered in Bangladesh(3). Genetics can play an important role in the conservation of endangered species. Since tiger populations are isolated into fragmented habitats, inbreeding and genetic drift may lead to decreased fitness and fertility, ultimately reducing the size of small populations further. Phenotypes such as deficits in fitness and fertility are detectable when the population is already affected. On the other hand, the genetic structure of a population can be determined beforehand and potential future status

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(e.g. inbreeding depression) of the population can be predicted. Therefore, monitoring the Bengal tiger population using molecular genetic tools will be helpful in the conservation of tiger species[4].

Different molecular markers are used for genotyping the populations. Examples include RAPD, RFLP, microsatellite markers, and sequences of selected mitochondrial and nuclear genes. Phylogeny, taxonomy, phylogeography, and demographic history of tiger species have been studied using molecular techniques, including mtDNA and microsatellite genotyping[4,5]. Among the mitochondrial genes, *CO1, 16S rRNA, 12S rRNA,* and *cytochrome b* gene sequences are generally analyzed. Particularly, *cytochrome b* gene sequence is vital in the taxonomic and phylogenetic study of the tiger.

DNA extraction from invasive tiger samples is extremely challenging because of the danger it poses, low abundance and elusive behaviors. Since killing or disturbing such an endangered animal species is highly objectionable, non-invasive genotyping has been developed as the preferred way to study a tiger's genome. Non-invasive samples include hair, scat, scent, etc., which can be obtained without killing or harming the organism. DNA is extracted from such a sample. Subsequently, genetic analysis become possible. Fecal samples (scat) has been used in many studies for DNA extraction, and its utility in genetic studies has been well reported[5-8]. Fecal hairs are also used as a source of genetic material[9]. To extract better quality DNA from feces, special care is required to avoid DNA degradation, the presence of PCR inhibitors, and DNA contamination. Recently, scent DNA has also been used in tiger conservation research[10]. There are commercial kits for fecal DNA extraction, but manual extraction is preferred due to low costs and flexibility to modify based on the conditions. There are reports on tiger genetics from India and Nepal though nothing could be retrieved about the genetics of the Bengal tiger from Bangladesh. So, as a beginning of tiger genetics, non-invasive genotyping from scat samples of *P. tigris* has been attempted from Bengal tiger captive at Dhaka Zoo. Then, a fragment of the *cytochrome b* gene has been sequenced using manually extracted DNA as a template.

Materials and Methods

Sample collection: The scat sample of Royal Bengal Tiger (*Panthera tigris* ORDER: Carnivora, FAMILY: Felidae) was collected from Dhaka Zoo (Mirpur, Dhaka, Bangladesh). After collection, scat samples were brought to the Genetics and Molecular Biology laboratory, Department of Zoology, University of Dhaka, and transferred to 95% ethanol and kept at room temperature for future use.

DNA extraction: Fecal DNA of the tiger was extracted mainly following previously published protocol with modifications[11]. Three samples were used for DNA extraction. Scat samples (1.5 g each) were vortexed and washed in 5 ml ethanol for 1 minute. Then, the samples were centrifuged for 8 min, and the supernatant was discarded. The washing

step was repeated with 5 ml TE (10 mM Tris, 1 mM EDTA, pH 8), 3 milliliter TNE (10 mmol/l Tris-Cl, 0.5% SDS, 1 mmol/l ($CaCl_2$). Then, 50 µl proteinaseK (20 mg/ml) was added. The mixture was incubated at 55°C for 2 hours. The lysate was centrifuged for 1 min. The supernatant was transferred into a new 15 ml tube containing 3 g potato starch. The tube was vortexed for 1 min to suspend the starch completely. The suspension was incubated for 1 min at room temperature. The starch tube was centrifuged for 12 min to pellet the starch. The supernatant (600 µl) was transferred into 2 ml centrifuge tube containing 150 µl NaCl solution (3.5 mol/l) and CTAB solution (0.7 M NaCl, 10% cetyl trimethyl-ammonium bromide). Then, DNA was extracted twice using an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The lower phase of phenol-chloroform was removed and the remaining upper phase was centrifuged for 5 min at maximum speed. The upper 500 µl was transferred to a new tube, and an equal volume of isopropanol was added. The mixture was inverted for 1 minute, and isopropanol was discarded after 5 minutes of centrifugation. Then, the DNA pellet was washed with 500 µl of 70% ethanol. Alcohol was removed, and the DNA pellet was dissolved in de-ionized distilled water. DNA of cow (*Bos indicus*) was isolated from a tissue sample using a common methodology of the laboratory[(12)].

Amplification and sequencing: A region of mitochondrial *cytochrome b* gene was amplified using the forward and reverse primers, 5'ATAACCCCTCAGGAATGGTG3' and 5'TGGCGGGGATGTAGTTATCA3', respectively[(13)]. PCR temperature regimes were initial denaturation for 2 min at 96°C, then, 30 cycles of denaturation (at 96°C for 30 sec), annealing (at 57°C for 30 sec) and extension (at 72°C, 30 sec), and a final extension at 72°C for 5 minutes. The amplified products were run electrophoretically on 1% agarose gel. DNA bands observed on UV-transilluminator were photographed by a gel documentation system. PCR products were then submitted for sequencing. Sequences were subjected to NCBI nucleotide BLAST.

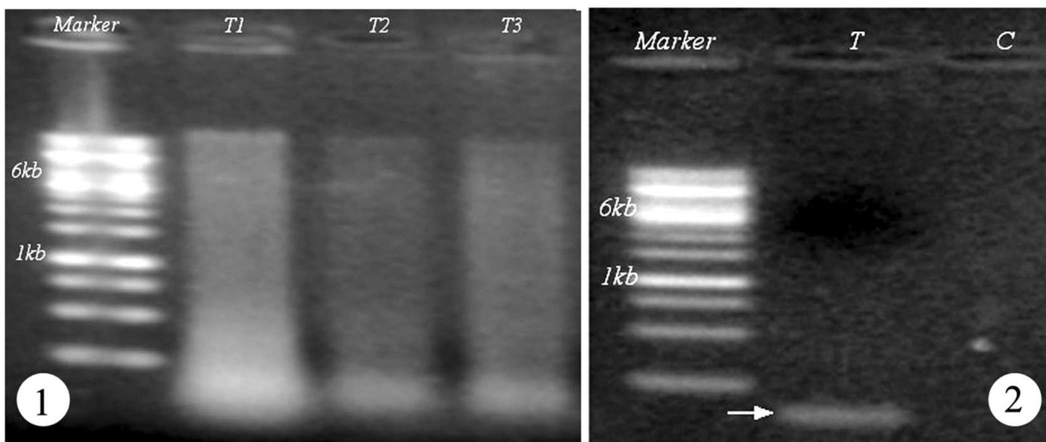
Results and Discussion

DNA was extracted manually from non-invasive samples without killing the organism concerned. The protocol for DNA isolation from fecal samples reported previously was modified for DNA extraction from the scat of the Bengal tiger (*P. t. tigris*) [(11)]. DNA extracted from all three scat samples of the Bengal tiger (*P. t. tigris*) showed its presence as bands and smears on agarose gel (Fig. 1). Moreover, cow DNA was used as a negative control in the PCR experiments while using tiger-specific primers.

The results of the PCR reactions proved that the DNA extracted was from the scat of the Bengal tiger (*P. t. tigris*). The size of the PCR product was expected to be 162 bp as indicated by primer alignment with available tiger mitochondrial gene sequences. As per expectation, a similar size band was observed in the agarose gel (Fig. 2). Use of species-

specific primers in the PCR reactions allowed to amplify only the *cytochrome b* gene fragment of the tiger, *P. t. tigris*.

Since the tiger is carnivorous and feeds on other vertebrates like a cow, buffalo, etc. Cow DNA has also been isolated and used as a template to check false positives in the PCR reaction. PCR with the same reaction condition and cow DNA template returned no product. No PCR product in case of negative control (template DNA from cow) further ensures the specificity of the particular region of the *cytochrome b* gene of the tiger. Thus, these results indicated that DNA extracted from fecal samples contained DNA of the Bengal tiger.



Figs 1-2: 1. DNA extracted from scat samples (T1, T2, and T3) of Bengal tiger visualized in 1% agarose gel. 2. PCR product of a *cytochrome b* gene region amplified from tiger DNA (*lane*-T) visualized in 1% agarose gel. No amplification was detected when cow DNA was used as a template (*lane*-C).

DNA isolation from fresh tissue and non-invasive samples such as scat have some differences. These differences are due to the nature of the samples. The presence of epithelial cells in the fecal samples is the main source of the DNA. In addition to the epithelial cells, a scat sample of tiger may contain chemical compounds as a by-product of digestion, which can subsequently be PCR inhibitors. So, removing such compounds during the process of DNA extraction has been a prime focus of the methodology. Previous reports on non-invasive DNA extraction from scat samples have taken the same strategy. To get better DNA from the scat samples of tiger in the present study, the modifications in the methodology are found to be useful.

Manual extraction protocol costs much less than commercial kits. However, Zhang *et al*.[11] isolated DNA from fecal samples of selected vertebrate species but not from that of tiger (*P. tigris*). Successful extraction of DNA from tiger scat samples in the present

study using the protocol of Zhang *et al.*[11] with slight modifications will be useful in studying the genetics of this animal.

Further, the sequence of the PCR product has confirmed that the template DNA was from the tiger. Nucleotide sequence subjected to NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) search returned with *cytochrome b* gene of *P. tigris* as topmost hits with 99% sequence similarity with that of other geographically distinct localities such as India, Thailand, and China. The difference only in few bases might be due to the individuality of the tiger sample. Previously, a sequence of *cytochrome b* gene fragments was used to differentiate field-collected scats of tiger and leopard before genotyping Amur tigers in Northeast China[14]. In another case, it was utilized in differentiating mainland tigers (Bengal, Chinese, etc.) taxonomically from that of Sumatran[15]. Thus, submission of *cytochrome b* gene sequence (Accession # KT726160) of Bengal tiger to publicly available GenBank database will be a valuable addition to the existing resources about tigers of different geographical localities. Since this is the first report of such work from Bangladesh, it would be a starting point of genotyping other mitochondrial regions and important nuclear genes of this magnificent animal of this locality. Thus, this study results could help in non-invasive genotyping, which could be instrumental to the conservation of the tiger species of the Sundarbans.

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