Short Communication

PHYLOGENETIC ANALYSIS OF CYTOCHROME B SEQUENCE OF *PANTHERA TIGRIS*

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ABSTRACT

Phylogenetic analysis of organisms not only offers crucial details on the genesis and evolution of genes, genomes, and species, but also helps with ecological and behavioural research while forecasting the magnitude and direction of future evolutionary trends in living things. In the present study, cytochrome b gene sequence of mitochondrial DNA obtained from captive Bengal tiger (Panthera tigris) from the Arignar Anna Zoological Park, Vandalur, was phylogenetically analysed with reference sequences of cytochrome b gene of fourteen sequences of the genus Panthera obtained from NCBI gen bank database. The similarities and differences between the different sequences were studied and the use of phylogenetic analysis as a forensic tool for investigation was discussed. The phylogenetic tool can also be used for pedigree checking where the parentage details are maintained and the place of origin of the species can be traced in case of samples seized during animal poaching.

Keywords: Phylogenetic analysis, cytochrome b, *Panthera* genus, forensics

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INTRODUCTION

The mitochondrial cytochrome *b* (cyt *b*) is a gene for which extensive sequence information is available for vertebrates. Details about its functional protein and biochemistry are well characterized. Hence, it has been highly sought after in establishing phylogenetic relationships due to its highly conserved nucleotide sequence among conspecifics (Lydeard and Roe 1997; Moore and DeFilippis 1997). Comparative analysis of cyt *b* gene among different vertebrate classes showed minimal genetic distances among congeneric and confamilial avians, herpeto and mammalian faunal species (Johns and Avise, 1998).

Starting with the prehistoric sabre-toothed cat (*Smilodon fatalis*), the scimitar-toothed sabre-toothed cat (*Homotherium serum*); the Pleistocene lion (*Panthera atrox*) to the current species of *Panthera* genus, the family felidae dominates the carnivorous guild in most ecosystems around the world (Van Valkenburgh, 1991; Janczewski *et al*., 1995).The largest of extant felid species, the tiger (*Panthera tigris*) once widely distributed in the central and south east Asia and numbering around 100,000 during 1900s currently faces risk of extinction due to fragmented habitats, poaching and illegal trade leading to critically lowered populations in its native habitats (Nowell and Jackson, 1996; Smith and McDougal, 1991; Kitchener and Dugmore, 2000). Of the eight widely accepted subspecies of tigers around the world, the Indian or Bengal tiger (*P. tigris tigris*) is found predominantly in the Indian subcontinent with estimated numbers of around 3200- 4500, of which the total count of tigers in India accounts for 2,967 (aged more than one year) individuals (Report, 2018).

The vast variation in modern tiger population and its evolution seems to have arisen due to clinal differences from the perspective of bio-geographical distribution and has significant impact on conservation efforts (Kitchener and Dugmore, 2000; Luo *et al.,* 2004). In India, PCR based assays of tiger specific cyt *b* gene are used for population estimation of tigers using faecal samples (Bhagavatula and Singh, 2006), while comparative and phylogenetic analysis of complete cyt *b* gene sequences reveal a distinct taxonomic identity to tigers (Cracraft *et al*., 1998).

Use of primers (Cao *et al.,* 2011; Naidu *et al.*, 2012) targeting cytochrome b region is widely practised for DNA amplification in forensic investigations for species identification. The sequence information of the amplified regions is essential for further analysis and comparison based on the reference data in GenBank and interpretation of Basic Local Alignment Search Tool (BLAST) search results. This study was undertaken to assess the phylogenetic relationship of a captive Bengal tiger with the GenBank sequences in order to assess the geographic place of origin of the animal.

MATERIALS AND METHODS

About 30-50g of tissue samples (liver and brain) from two tigers were collected during post mortems conducted in Arignar Anna Zoological Park, Vandalur, Chennai, during 2021. The tissue samples (in duplicate) were subjected to DNA extraction using DNeasy Blood and Tissue Kit following the procedure recommended by the manufacturer. Purity and concentration of DNA were measured by using NanoDrop spectrophotometer and DNA was stored at a concentration of 20ng/µl at -20°C for further analysis.

The tiger specific primers (Tigris-F: TTT ACG GTC ATG GCT ACA GCC and Tigris-R: GAC TGC TGC TAG GGC TGA GAC) designed by Cao *et al*., (2011) used for amplification of cytochrome b gene (cyt *b*) and synthesized from Eurofins Genomics India Pvt. Ltd., Bengaluru, was utilized for this study. Amplification was carried out in a 25µL reaction volume containing 20ng of template DNA, 100 µM each of dNTPs, 10pmole of primer, 1.5mM $MgCl_2$, and 0.5 units of Ampliqon (Cat. No. A180301) and 1xPCR buffer (10 mM Tris-HCl, ph 8.3 and 50 mM KCl). PCR cycling was performed in a thermal cycler (T100 thermal cycler, Bio-Rad) with a ramp rate of 4°C/sec. The PCR conditions were: initial denaturation at 94°C for 3 minutes, followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 72°C for 1 minute and primer extension at 72°C for 1 minute. The extension step at the 35^{th} cycle was held for 7 minutes. The PCR products were analyzed on 2% agarose gel electrophoresis by loading 10µL of PCR product into wells with 100Kb DNA ladder as marker. Bulk PCR was carried out with the sample where, 50µL of the product was electrophoresed and gel extraction of the product was carried out using MinElute® Gel Extraction Kit (50) (Qiagen, Germany). Cycle sequencing of gel purified cyt b PCR product was carried out in an automated DNA Sequencer by Eurofins Genomics India Pvt. Ltd., Bengaluru, and the sequence (named as MVC/WLS/2021/001) was analyzed for phylogeny test with the sequences of *Panthera* species from GenBank, using the Neighbour-Joining (NJ) based on the Kimura 2 parameter(K2P) model and Maximum likelihood (ML) method based on K2P model with the Neighbour-Joining Algorithm under Molecular Evolutionary Genetics Analysis (MEGA) 10.0 software program.

RESULTS AND DISCUSSION

Results of DNA purity and concentration (ng/µl) were recorded and plotted automatically. The purity of DNA ranged between 1.6 – 1.9. PCR amplification was carried out on the DNA samples with the tiger specific primer (Tigris-F and Tigris-R). Subsequent agarose gel electrophoresis revealed a 225 bp fragment, representing the cyt *b* region from all the samples (Fig. 1). The results were in concurrence with Cao *et al.*, (2011). The samples were further subjected to DNA sequencing (Sanger's technique). However, only one sample was qualified for sequencing and the results produced readable sequences (MVC/WLS/2021/001).

BLAST analysis of cyt b sequence verified the similarity (100%) of the sequence to the *P. tigris* .

The phylogenetic tree based on cytochrome b sequence of 15 *Panthera* genus (tiger, leopard and jaguar) was generated by using the neighbor-joining algorithm, test of phylogeny in the bootstrap method with 1000 replicates, using the MEGA 10.0 software program. The phylogenetic analysis of cytochrome b gene revealed a formation of two clades (Fig. 2). Clade A had five different clusters (sub groups) and clade B had six different clusters, all belonging to the *Panthera* genus from Genbank, NCBI databases. The MVC/WLS/2021/001 sequence belonging to *P. tigris* was grouped in the clade A. It was 100% similar to the *P. tigris* sequence (EF392684 from India), 90% similar to another *P. tigris* sequence (DQ151551 from USA) and 49% similar to *P. tigris tigris* (AF053053 from USA), *P. tigris sumatrae* (AF053054 from USA), *P. pardus orientalis* (AB817079 from Japan) and *P. pardus saxicolor* (MK028952 from UK). The other group comprising of *P. tigris amoyensis* (AY452089 from China), *P. onca* (KP202264 from USA), *P. pardus* (KP001507 from The Netherlands; MH588627 from Germany; MH588623 from Germany), *P. tigris corbetti* (JF357972 from Australia), *P. pardus japonensis* (KJ866876 from China) and *P. pardus orientalis* (KX655614 from China) formed clade B. In clade A, there were five clusters and the first three clusters correspond to the Asian mainland tigers and Bengal tiger. The cluster four corresponds to Sumatran tiger and cluster five belonged to leopards.

The various species of extant tigers (*P. tigris*) exist in different bio-geographical regions as six major subspecies *viz*., The Bengal tigers (*P. t. tigris*); Northern Indochinese tigers (*P. t. Corbetti I*); Amur tigers (*P. t. altaica*); Southern Indochinese tigers (*P. t. corbetti II*) and Sumatran tigers (*P. t. sumatrae*), each subspecies occurring as a result of limited gene flow and hence significant variation in genetic structure especially mtDNA, geographical isolation and allopatry (O'Brien and Mayr, 1991; Avise, 1990; Kitchener and Dugmore, 2000; Seidensticker *et al,* 1999). An average pair-wise sequence similarity of 94-99% has been recorded using maximum parsimony and neighbor-joining phylogenetic analysis of cyt *b* gene among 20 extant felid species (Masuda *et al.,* 1996). Similarly, phylogenetic analysis using mitochondrial cyt *b* gene has helped in the reconstruction of phylogenetic tree and revealed the distinct taxonomic position of extinct cave lion (*P. leo spelaea*) among the extant species of lions (Burger *et al.,* 2004).

The phylogram also showed occurrence of sub-groups within a cluster which indicates existence of more genetic similarity and lower genetic distance between the species of a sub-group than the other members of the particular lineage. The sample under study was compared with the known sequences of various related felids and it was concluded as Asian mainland tiger. The phylogeny study can be used for confirmation of forensic studies where the sample is of unknown origin. The sequence results of the sample can be blasted in GenBank to assess the homogeneity between the species and the

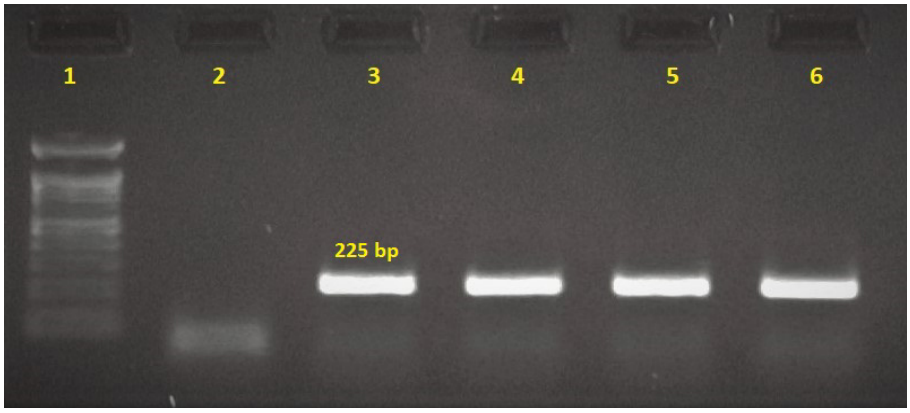

Fig. 1. Agar gel electrophoresis of PCR products amplified using tiger-specific primer Tigris-F and Tigris-R. Lane 1-100 bp ladder, lane 2-blank, lanes 3-4 tiger 1 and lanes 5-6 tiger 2.

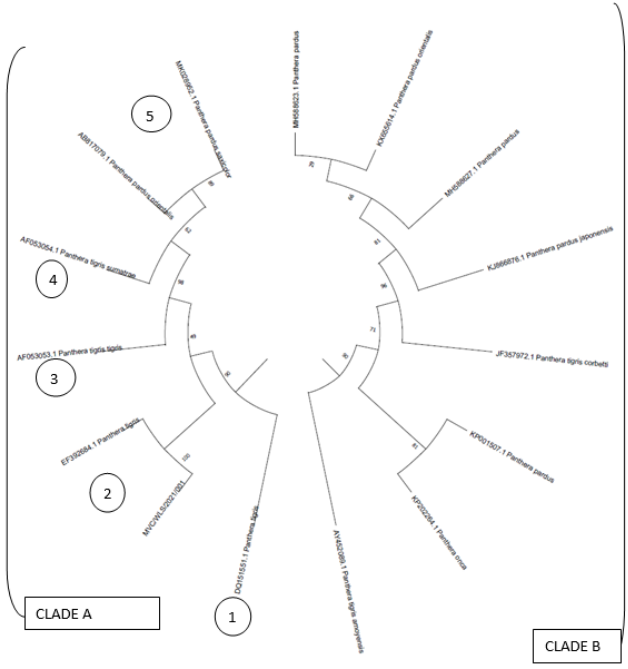

Fig. 2. Neighbor-Joining phylogenetic tree *Panthera tigris* and comparison species based on cyt *b* gene with 1000 bootstrap replicates

result can be analyzed with the phylogenic evaluation. In case of wild animals maintained under captivity, the study can be used to ascertain the parentage details and for poached animals the origin can be ascertained based on the sequences available in the GenBank.

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