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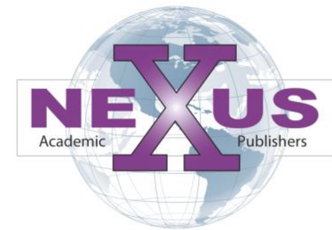

Research Article

Comparison of D–Loop Gene for Subspecies Confirmation of Tigers (*Panthera Tigris*)

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ABSTRACT

Tiger (*Panthera tigris*), the biggest cat species and the top predator has fallen into endangered category. In the present study, we confirmed the subspecies of tiger with help of DNA based methods. The mitochondrial control region gene (D–loop) sequence was utilized for this purpose. DNA was extracted from tiger blood sample originally obtained from mini zoo of Rohtak, Haryana. The D–loop sequence was amplified and submitted to GenBank with accession number HM362438. Based on D–loop sequence identity and phylogenetic analysis *Panthera tigris tigris* (Indian tiger) was differentiated from other *Panthera tigris subspecies* (*Panthera tigris amoyensis*, *Panthera tigris corbetti*, *Panthera tigris sumatrae* and *Panthera tigris altacia*) from different parts of the world. The present report also describes an easier, quicker and non hazardous protocol for the isolation of genomic DNA from tiger blood.

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INTRODUCTION

The global adult tiger population as per Global Tiger Recovery Program (2012) has been estimated to be only 4,000 as compared to 1,00,000 in the beginning of this century (Chundawat et. al., 2012). The demographic distribution of tigers shows a great shrinkage in their range declining to only 7 percent of historic range (Dinerstein et. al., 2007). Forensic identification of tiger species can be carried out using various methods. However, molecular methods provide a direct and confirmatory approach to identify and distinguish among closely related species or subspecies (Gupta et. al., 2005). Also, the need to stop the ongoing havoc of poaching and illegal trade of tiger body parts rises the importance of DNA based molecular approach. It involves the use of both nuclear as well as mitochondrial (*mt*) DNA based markers. However, *mt* DNA markers have been reported to be more efficient than nuclear markers for identification and authentication of species (Rastogi et al., 2007), as the rate of evolution of *mt* genome is much faster than nuclear genome thereby helping in differentiation of closely related species (Brown et al., 1979; Kitpipit et. al., 2012). Phylogenetic relations can be easily drawn on the basis of the *mt* gene studies and thus, can be compared with the distant and near tiger relatives. The mt DNA control region (D–loop) analyses showed a central conserved region, two hypervariable segments along with size and sequence heteroplasmy in five species of the genus Panthera (Jae–Heup, et al., 2001). The control region of mt DNA (D–loop) was used successfully for species identification of five different species viz., red deer (Cervus elaphus), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), mouflon (*Ovis aries musimon*) and wild boar (*Sus scrofa*) using hair samples (Parkanyi et al., 2013). The mt DNA control region (D–loop) was also used for phylogeographic patterns and evolution studies of two Neotropical cats viz. ocelot (*Leopardus pardalis*) and margay (*Leopardus* wiedii) (Eizirik et al., 1998).

Blood has been extensively used for obtaining genomic DNA (gDNA) for population studies; however, quicker, safer and easier protocols are needed, to apply them for larger population structure. Therefore, the present study was also planned to develop an easier and non – hazardous protocol for isolation of DNA from blood after doing certain modifications in the chelex gDNA extraction protocol (Walsh et. al., 1991).

MATERIAL AND METHODS

Sample Origin

The blood sample from tiger (named Saurabh) was received from Mini zoo of Rohtak District of Haryana state. The blood sample was sent in EDTA anticoagulant in the teaching veterinary clinics of the college for routine haemoprotozoan investigation.

Genomic DNA Extraction

The extraction of gDNA was done using standard protocol with certain modifications (Walsh et. al., 1991). Briefly, RBC lysis buffer containing 17mM Tris HCl (pH 7.65) and 140 mM NH_4Cl, was added to the sample in the ratio of 1:2 and kept for 15 min at room temp. Centrifugation was done at 8,000 rpm for 20 min. RBC lysis step was repeated until a white pellet was obtained. The white pellet was dissolved in 100µl Lefton's buffer (25mM Tris HCl, pH 7.5, 100mM EDTA and 1% SDS), mixed and kept for 15 min at room temp. To the lysate 200µl of 5% chelating resin, 2.5µl of

proteinase K (10mg/ml) and 4.5 μl of DTT (2M) were added. This solution was incubated at 56°C for 45 minute with intermittent shaking. After incubation, the enzyme was inactivated in boiling water bath for 10 minute. Supernatant was collected in a fresh eppendorf tube after centrifugation at 12,000 rpm for 1 minute.

Quantification of gDNA

The extracted gDNA for further processing required more reliable values measured with a high fidelity spectrophotometer. The quality and quantity of gDNA was assessed using Picodrop™ high fidelity UV spectrophotometer (Astranet systems Ltd. ®, U.K.) as per standard procedure. In Brief, 1 μl of purified DNA sample was taken in the disposable UVpette® tips (Astranet systems Ltd ®, U.K.). The optical density of gDNA sample was determined at A_{260} and A_{280}, keeping NFW as blank. The quantity of the gDNA was measured by using the standard reading of 1 O.D. at A_{260} for 50μg/ml. The reading was obtained in ng/μl.

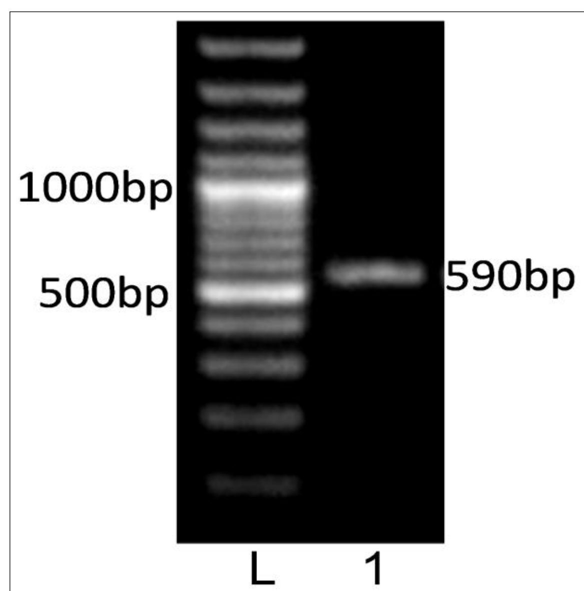

Figure 1: D–Loop gene based specific amplification of Tiger blood samples from (Saurabh, Acc. Number HM362438) showing amplicon of 590bp; Lane L: DNA Marker 100bp; Lane 1: Saurabh.

PCR Amplification

The tiger species specific primer pairs (Forward 5'TAGCCCCACCATCAGCACCCAAAGC3' and Reverse 5'AATGGGCCCGGAGCGAGAAGAGGTA3') targeting hypervariable D–loop region of *mt* DNA was used to amplify the gDNA (Zhang et. al., 2006). The forward primers correspond to positions 16263 – 16287 nt and reverse primer 16875 – 16899 nt of complete mtDNA sequence of *Felis catus* (Lopez et al. 1996). The PCR reaction was conducted using 2U of Taq polymerase (Fermantas, USA), 200 μM of dNTP, 1.5 mM $MgCl_2$ and 20pM of forward and reverse primers each in thermal cycler (iCycler Biorad, USA). The thermal condition was set as initial denaturation of 95ºC for 5 minute, followed by 30 cycles of denaturation (94ºC for 40second), Annealing (67ºC for 50second) and Extension (72ºC for 55second). The final PCR extension was set at 72ºC for 10 minute. The PCR product obtained was checked by agarose gel electrophoresis in 1.5% agarose gel using ethidium bromide stain.

Sequence Data Analysis

The D loop PCR Product was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) in ABIPRISM 3130xL Genetic Analyzer™ machine in our laboratory. The sequence was subjected to BLASTn search in GenBank data base for confirmation as Tiger D–loop (control region) sequence. The sequence data of D–loop was subjected to percent identity matrix calculation with sequences of different sub–species of tiger family using Bioedit v 7.2.3 (Hall, 1999). The neighbor joining tree was also drawn based on percent identity and bootstrap values (with 1000 replicates) using p–distance algorithm of MEGA 5.2 (Tamura et.al, 2011) software.

RESULTS

The genomic DNA from blood sample was extracted using chelex based method. The gDNA was found to be 143.16 ng/μl after measuring by Picodrop™ UV spectrophotometer. The gDNA was then subjected to D–loop gene specific PCR with standardized primer (Zhang et. al., 2006). The species specific D–loop gene primer pair yielded a PCR product of 590bp (Figure 1). The PCR amplicon of D–loop region was sequenced in our laboratory. The sequence data was submitted to GenBank data base and an accession number HM362438 was assigned to it. The sequence data of Saurabh (Accession number HM362438) showed more than 99% identity with *Panthera tigris tigris* after BLASTN+2.2.28 search (Zhang et al., 2000) in GenBank database. Pairwise nucleotide identity of Saurabh (Accession number HM362438) with other *Panthera tigris* subspecies were also calculate using Bioedit v 7.2.3 software (Hall, 1999) (Table 1). A D– loop gene based neighbor joining tree (NJ Tree) of Saurabh (Accession number HM362438) with other subspecies of tigers from different parts of the world was constructed using Mega 5.2 software (Tamura et al., 2011) (Figure 2).

DISCUSSION

Tiger subspecies confirmation can help in genome conservation in a better way and characteristics of different tiger subspecies can be maintained in the gene pool. The method that has been developed is easy, quick and most importantly requires few micro–liter of blood to yield a significant amount of DNA even from clotted and degraded blood sample, thus allowing further genetic analysis. As the *mt* DNA accumulates mutation faster than the nuclear DNA, it is helpful in studying the genetic diversity among the Indian tigers. The sequence analysis of D–loop from Saurabh tiger revealed that it was very closely related (>99% nucleotide identity) to *Panthera tigris tigris* from China and United Kingdom. However, it was also 98.9 to 97.6% identical with other *Panthera tigris* subspecies (*Panthera tigris amoyensis*, *Panthera tigris corbetti* and *Panthera tigris sumatrae*) from China, United Kingdom and USA. It showed the close relationship of *Panthera tigris tigris* subspecies and the common line of its evolution. However, it is distinct from other *Panthera tigris* subspecies from different parts of the world. Moreover, it was distantly related (only 40.7 to.

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Sr. No.	Panthera tigris Sequences	% identity with Indian Tiger (Saurabh, Acc. Number HM362438)
1	Panthera tigris tigris/HM362438/India	100
2	Panthera tigris tigris/AY452116/China	99.4
3	Panthera tigris tigris/AY452114/China	99.4
4	Panthera tigris tigris/JF357968/United Kingdom	99.0
5	Panthera tigris tigris/JF357967/United Kingdom	99.0
6	Panthera tigris amoyensis/HM589215/China	98.9
7	Panthera tigris corbetti/JF357971/United Kingdom	98.7
8	Panthera tigris sumatrae/JF357969/United Kingdom	98.5
9	Panthera tigris/DQ151550/USA	98.5
10	Panthera tigris/EF551003/China	97.6
11	Panthera tigris altaica/AY452113/China	40.7
12	Panthera tigris altaica/FJ460708/Canada	40.4
13	Panthera tigris altaica/FJ460709/Canada	40.4
14	Panthera tigris altaica/FJ460707/Canada	40.2
15	Panthera tigris altaica/FJ460710/Canada	39.8

Table1: Percent nucleotide identity of Indian *Panthera tigris tigris* with other Tiger species.

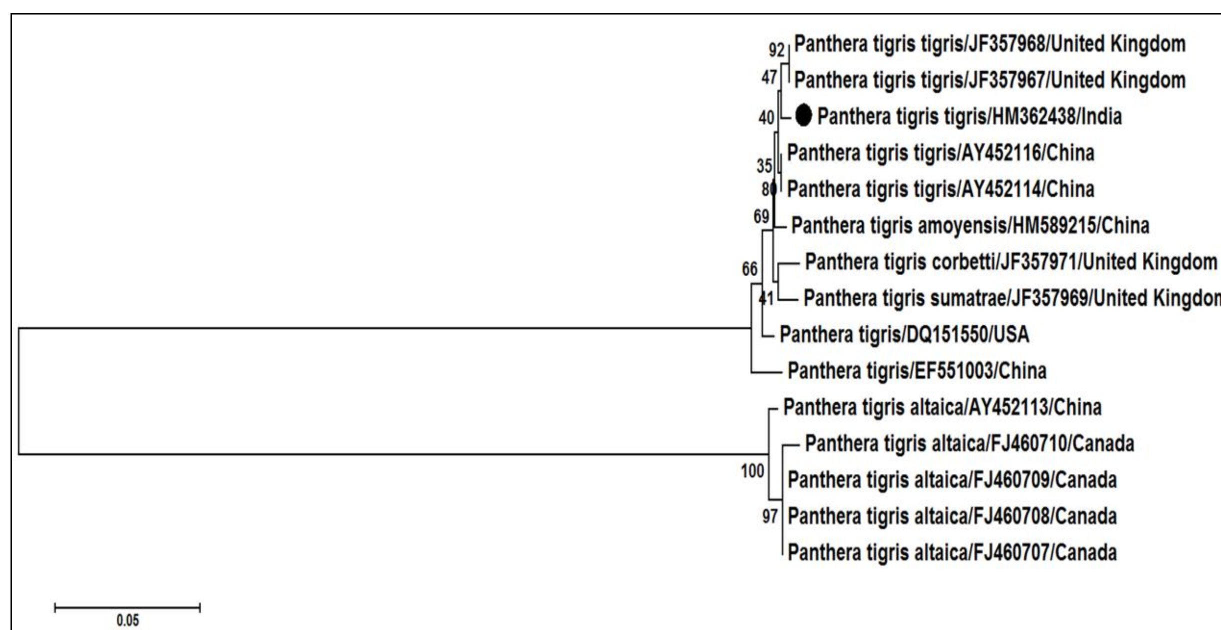

Figure 2: D–loop gene based NJ Tree of Indian Tiger (Saurabh, Accession number HM362438). Tiger in comparison with other *Panthera tigris* sub–species of tigers from different parts of world. ●= Used in present study.

39.8% nucleotide identity) to *Panthera tigris altaica* from China and Canada. The phylogenetic analysis also showed that Indian Tiger Saurabh (Accession number HM362438) formed a very close cluster with *Panthera tigris tigris* from China and United Kingdom. However, it was also closely related with different *Panthera tigris* subspecies (*Panthera tigris amoyensis*, *Panthera tigris corbetti* and *Panthera tigris sumatrae*) from China, USA and United Kingdom. The *Panthera tigris altaica* from China and Canada formed a separate cluster and were distantly related to *Panthera tigris tigris*.

The D–loop gene along with other mitochondrial gene cytb can be used for the species differentiation of tiger and other wild animals. The species differentiation between Indian wolves and other Canid species were done using D–loop, 16S rRNA and cyt*b* gene sequence (Aggarwal et. al. 2007).

CONCLUSION

Tiger is an endangered species in India and throughout the world. To control poaching, illegal trade of tiger body parts and pure line breeding in captive condition the proper species identification up to subspecies level is essential. In present study the control region of mitochondrial (D–loop) region was amplified, sequenced and compared with other Tiger subspecies for sub species confirmation. Our analyses showed that based on D–loop sequence *Panthera tigris tigris* (Indian tiger) can be differentiate from other Panthera subspecies.

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CONFLICT OF INTEREST

We declare no conflict of interest.

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