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Use of molecular-based approach in resolving subspecies ambiguity of the rescued tiger cubs from Arunachal Pradesh, India and their relationship with other population

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Genetic approach is often suggested for resolving taxonomic ambiguities in areas where there have been overlaps in distribution of a species or subspecies. The Northeast part of India is one of the identified biodiversity hotspots, having the junction of Palearctic and Indomalayan realms with dense canopy forests and rugged terrains. The distribution range of two tiger subspecies, i.e. Bengal tiger and northern Indo-Chinese tiger, overlaps with each other in this region. The government authorities rescued three tiger cubs from Angrim Valley Village, Arunachal Pradesh, Northeast India in November 2012. We carried out a comprehensive genetic study on the rescued cubs to elucidate their subspecies status and determine their relationship with the remnant tiger populations. Our findings based on 3661 base pair of mitochondrial DNA sequence spanning across six mitochondrial genes (ND1, ND2, ND5, ND6, 12S rRNA and CytB) and non-coding control region (CR) suggest the Bengal tiger ancestry of the rescued tiger cubs. Further, comparison of mitochondrial haplotype with other Bengal tiger populations reveals that the haplotype reported for the rescued cub is novel and has close affinity to the northeast tiger populations of India. However, detailed population assignment to infer the source of origin was not possible due to lack of genetic data for all Bengal tiger populations. The present study attempts an assessment of tiger status that has resulted in identification of another tiger occupied landscape in India (Dibang Tiger Reserve).

Keywords: Bengal tiger, DNA sequencing, single nucleotide polymorphism, subspecies.

HUMAN-INDUCED environmental changes have deteriorated habitat quality and quantity in different ecosystems worldwide and have driven most of the wildlife towards extinction^{1–3}. The larger species experience aggravated effects of human-induced environmental changes as

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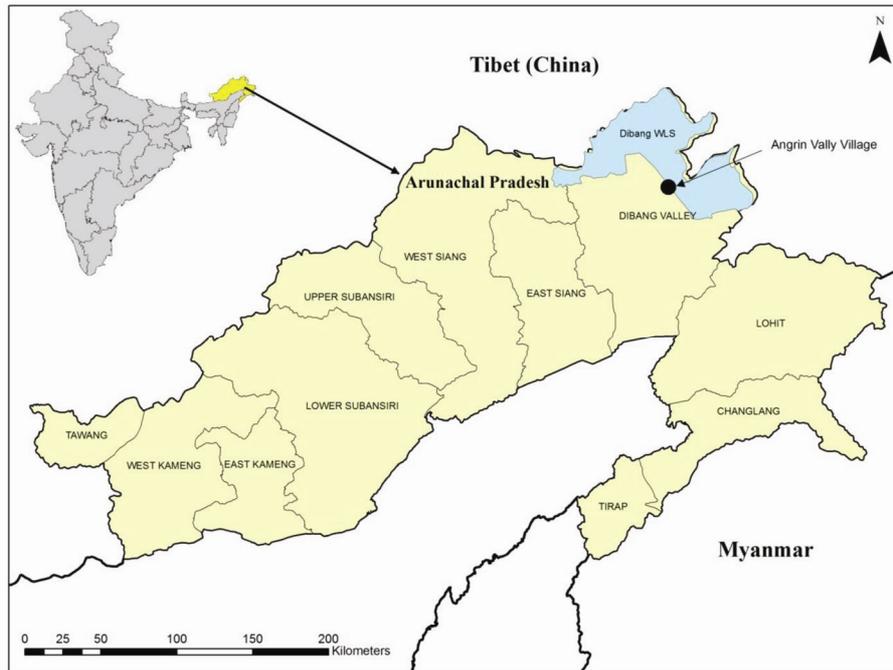


Figure 1. Location of the site (Angrim Valley) from where the tiger cubs were rescued.

compared to smaller species and thus require species-based conservation approach⁴.

Tiger (*Panthera tigris*), the magnificent apex predator of Asia, is one such species that experienced drastic population decline (>95%) and distribution range collapse (>93%) over the past few decades⁵⁻⁷. Today, most (>50%) of the wild tigers are surviving in the forests of India⁸. Since the beginning of the 20th century, the number of tigers has declined rapidly in India. Uncontrolled hunting, habitat loss and prey-base depletion reduced their number to less than 2000 individuals by 1973, thus impelling the government to undertake necessary measures to revive tiger populations⁹. The first was the ban on tiger hunting (in 1970), followed by providing legal protection under the Wildlife (Protection) Act in 1972, designating tigers as the national animal (1973) and initiation of world's largest conservation project (Project Tiger, 1973)¹⁰. Despite all efforts, tigers are still classified as 'endangered' as the measures undertaken failed to prevent their poaching and habitat encroachment.

Northeast India is one of the most diverse landscapes concerning communities, species, and endemics as it is the meeting zone of the Palearctic and Indomalayan realms¹¹. The region is now identified as the biodiversity hotspot with cross-border contiguity with the neighbouring countries like China and Myanmar. The forests of northeast India are dense (tropical evergreen) with rugged mountainous terrains and inhospitable conditions¹². Thus, a limited number of status surveys were undertaken in the region leading to paucity of data on species distribution. The region has the highest conservation significance

among all tiger occupied landscapes as it is situated at the junction of overlapping distribution ranges of two tiger subspecies, i.e. Bengal tiger (*Panthera tigris tigris*) and northern Indochinese tiger (*Panthera tigris corbetti* I)^{5,13}.

In November 2012, the government authorities rescued three tiger cubs from Angrim Valley Village, a human settlement around Dibrang Wildlife Sanctuary (DWS) of the northeastern province of India (Figure 1). The source of origin of the rescued cubs was unclear as there was no prior documented record of tiger presence in DWS and adjoining forests up to that time^{14,15}. Thus, there was an immediate need for detailed phylogenetic examination of the rescued cubs, as such information would help in formulating better rehabilitation policies or in developing trans-boundary conservation strategies.

The potential of mitochondrial DNA has been widely utilized for reliable species and subspecies identification, assignment of confiscated articles to the source of origin and to understand the phylogenetic relationship¹⁶⁻¹⁹. Luo *et al.*⁵ studied all extant tiger populations genetically and identified key subspecies specific nucleotide positions in the mitochondrial genome. Subsequently, their findings were utilized in elucidating subspecies status of extinct Caspian and Korean tigers and to examine the genetic ancestry of captive tigers worldwide^{18,20,21}. Mitochondrial DNA fragments were also analysed for several tiger populations in India to understand the genetic relationship, gene-flow and phylogeography^{6,19}.

Since nothing was clear about the origin of these tiger cubs, we performed the study systematically, i.e. first, a detailed subspecies identification was done, as the

rescued site was within the overlapping range of two tiger subspecies and secondly, population assignment was assessed to identify the most genetically related population. The present findings will hopefully have broader applications in formulating trans-boundary conservation strategies.

The present study was undertaken with permission and request from the Arunachal Pradesh Forest Department and the National Tiger Conservation Authority. This permission was received in writing on 9 January 2013. The sample was provided by the Arunachal Pradesh Forest Department and hence no animal handling was done during the study.

Blood sample was obtained from one of the rescued cubs (considering that all belonged to one maternal lineage) in EDTA containing vacutainer under sterile conditions. Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer protocols. We amplified and sequenced ~4 kb DNA covering six mitochondrial genes (ND1, ND2, ND5, ND6, 12S rRNA and CytB) and non-coding control region (CR) that were established as variable and informative^{5,20-22}. Polymerase chain reaction (PCR) amplifications were performed on ABI 9600 Fast (Applied Biosystems, Switzerland) thermocycler in a total reaction volume of 10 µl following Luo *et al.*⁵. The amplified products were cycle-sequenced, cleaned and finally analysed on 3130 genetic analyser (Applied Biosystems, USA). The quality of DNA sequence was determined using Sequence Analysis Software Package (Applied Biosystems) and validated using Sequencher 4.7 (Gene Codes Corporation, USA).

The genetic ancestry of rescued cubs was determined by comparing their mitochondrial gene data with published tiger haplotypes^{5,6}. Since there was variation in total length of mtDNA sequences reported by Luo *et al.*⁵ and Mondol *et al.*⁶, we carried out separate analysis of independent datasets. In total, we analysed and compared 3661 bp sequence with Luo *et al.*⁵ and 1250 bp with compatible sequence data of Luo *et al.*⁵ and Mondol *et al.*⁶.

All the different sets of compatible sequences were aligned, and multiple sequence alignment (MSA) performed using Clustal W as implemented in BioEdit v 7.0.9.0 software²³ and then manually edited to get the best-aligned sequences. The phylogenetic trees were generated based on neighbour-joining (NJ) and maximum likelihood (ML) methods for all the sets of aligned sequences using the bootstrap value of 1000 iterations as implemented in MEGA v. 6 (ref. 24). Model generator²⁵ software was used to determine the substitution model of DNA sequence evolution that had the best-fit of mitochondrial sequence data. Model generator selects best-fit model under the Akaike Information Criterion (AIC)²⁶. The chosen model is the one that minimizes the Kullback–Leibler distance between the model and the reality.

The model of evolution selected by model generator corresponded to HKY²⁷ for both sets of sequences.

We obtained a total of 3661 base pairs of good quality (Q value >50) sequence for the tested sample with 18 subspecies-specific nucleotide variations⁵. We found unique single nucleotide polymorphism (SNP) variations at positions 14523 (ND6 gene) and 16429 (non-coding CR) corresponding to complete reference *Felis catus* mtDNA sequence (Table 1). Sequence alignment showed the presence of Bengal tiger characteristic subspecies-specific nucleotide variation in ND2 (5050 and 5533) and ND6 (14618) genes (Table 1). Alternately, no subspecies characteristic SNP of other tiger subspecies was found in the tested sample. In phylogenetic tree (ML), the rescued cub formed the monophyletic group with rest of the Bengal tiger haplotypes (Figure 2). Based on the above findings, we conclude that the rescued cubs belonged to Bengal tiger subspecies (*Panthera tigris tigris*). Further comparison of mtDNA sequences of rescued tiger cub sample with other Bengal tiger sequences⁶ from northern, northeast and peninsular India revealed that the haplotype was unique and shared close genetic ancestry with TIG25 haplotype (Figure 3).

Indian tigers have a great role to play in the revival of global tiger population with more than 60% of extant genetic diversity⁶. Among all Bengal tiger populations, the tigers of northeast India are of higher conservation significance as the region marked the entry route of tigers in the Indian subcontinent, thus presumed to retain higher genetic diversity^{19,28}. Our genetic findings confirmed the

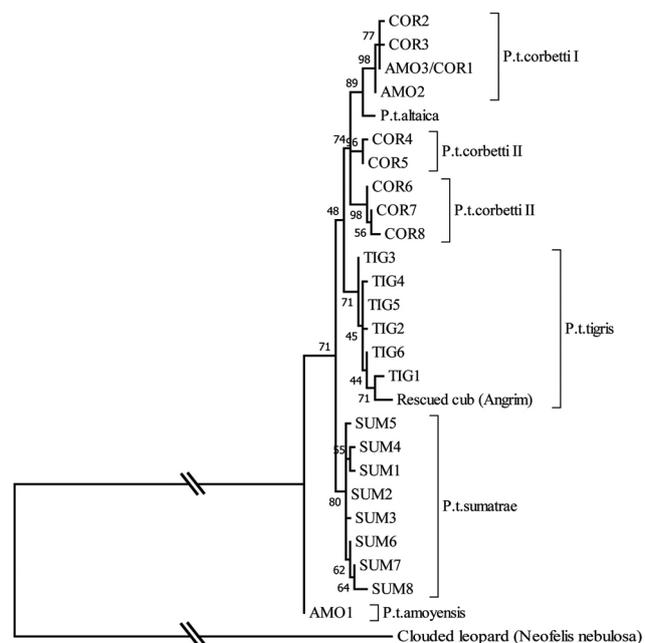


Figure 2. The phylogenetic positioning of rescued cubs examined with published tiger subspecies haplotypes⁵ using the maximum likelihood method based on the Hasegawa-Kishino-Yano model.

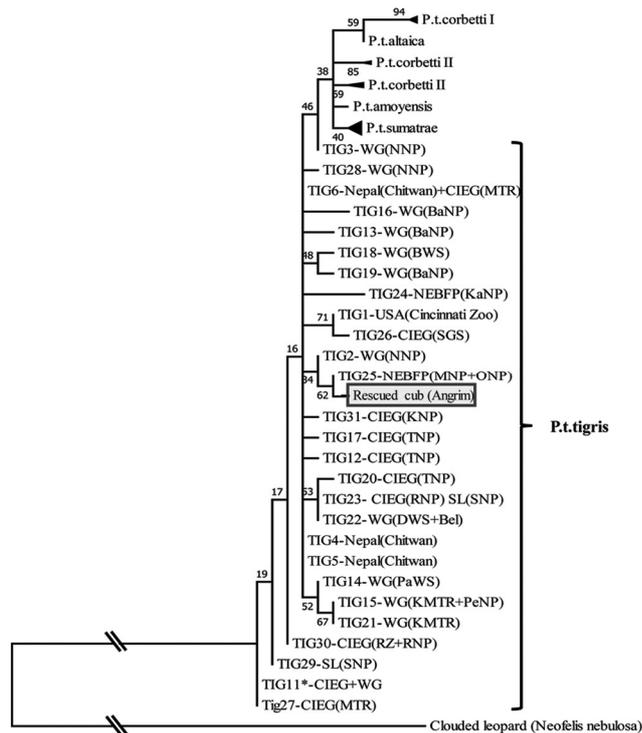


Figure 3. Phylogenetic tree created from four mtDNA genes (Cytb, ND2, ND5 and CR) depicting genetic relationship between rescued tiger cub and all haplotypes found in Bengal tiger populations⁶. [Haplotype found in several populations of CIEG and WG landscapes. WG, Western Ghats; CIEG, Central India and Eastern Ghats; NEBFP, North East and Brahmaputra Flood Plains; SL, Sunderbans Landscape; NNP, Nagarhole National Park; BaNP, Bandipur National Park; BWS, Bhadra Wildlife Sanctuary; KaNP, Kaziranga National Park; SGS, Satkosia Gorge Sanctuary; MNP, Manas National Park; ONP, Orang National Park; KNP, Kanha National Park; TNP, Tadoba National Park; RNP, Ranthambhore National Park; SNP, Sunderbans National Park; DWS, Dandeli Wildlife Sanctuary; Bel, Belgaum (Karnataka); PaWS, Parambikkulam Wildlife Sanctuary; KMTR, Kalakkad Mundanthurai Tiger Reserve; PeNP, Periyar National Park; RZ, Raipur Zoo (Chhattisgarh) and, MTR, Melghat Tiger Reserve.]

subspecies status of rescued cubs (Bengal tiger), but we were unable to assign them at the sub-population level with precision due to paucity of genetic data. Genetic closeness with reported TIG25 haplotype suggested that the rescued cubs originated from a population of north-east India.

There is limited information available about tiger distribution in northeast India due to logistical and operational constraints. Being densely forested with a rugged terrain with low tiger densities, the population monitoring in northeast India using traditional approaches like sign survey, pugmark or camera traps is difficult^{12,29,30}. Therefore, we suggest the use of non-invasive genetic sampling (NGS)-based status surveys in the forests of northeast India.

Tiger rescue is common in India considering enough anthropogenic pressure and human settlements surrounding the protected areas. However, the present reported

case is unique as it was the first reported incident of tiger presence in one of the eastern-most provinces of Arunachal Pradesh, thus having a remote possibility to be of the northern Indochinese subspecies. After being established the genetic ancestry as ‘Bengal tiger’ of rescued cubs from an unknown population, the State Forest Department initiated a pilot project (subsequently during All India Tiger Monitoring 2014) to assess tiger presence in DWS and adjoining forests which were pending for a long time. An ecological survey undertaken with camera-trap and photographs suggested the presence of tigers in DWS.

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Analysing the black polished pottery of proto-historic-early historic India

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The development of ceramic technology in India can be traced back to a hoary past. Highly sophisticated potteries like black slipped ware (BSW), black-and-red ware (BRW), painted grey ware, grey ware and northern black polished ware (NBPW) were present in proto-historic and early historic times (circa 2000–300 BCE). The present communication aims to throw light on the technology of one of the important wares, viz. BSW especially from sites located in the Vindhya–Ganga region. Through analytical examination of the ware, the authors propose to fill in a lacuna in the field of ancient ceramics.

Keywords: Black slipped ware, energy-dispersive X-ray spectroscopy, firing technique, proto-historic, scanning electron microscopic study.

THE black slipped ware (BSW) is a black coloured glossy ware first reported by Lal¹ in pre-northern black polished ware (NBPW) deposit from excavations at Hastinapur. Initially it was variously referred by archaeologists as black polished ware¹ or black slipped ware or burnished black ware. Since then this pottery has been recovered in different contexts. The black-and-red ware (BRW), which generally co-exists with BSW has received greater attention of scholars while in-depth studies on BSW are few and far between, despite the fact that BSW is stated to have contributed to the make-up of the famous NBPW. The present discussion on BSW is focused primarily on its analytical study. It also tries to compare BSW with NBPW in fabric, form and finish.

BSW as its nomenclature suggests is a pottery which contains a black and smooth polished surface. BSW is carefully turned on fast wheel, treated with a smooth black slip, both on the inner and outer surface and possibly also burnished for a glossy slip. It has a medium to fine grey or blackish grey core². The quality of the ware exhibits mastery in the pottery making technique. Like the other grey or black wares of its time, the most common shapes in this ware are bowls, dishes, basins and vases. Sometimes platter and miniature pots were also found at a few sites (Figure 1).

BSW has wide distribution both in time and space right from chalcolithic/pre-NBPW levels to the closing centuries of the 1st millennium BCE (second millennium BCE to 300–200 BCE). Geographically BSW commands an extensive distribution area extending from Manda

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