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Application of forensic entomology in carcass examination of royal Bengal tigers (*Panthera tigris tigris*) in Madhya Pradesh

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Abstract

Royal Bengal Tiger comes under flagship species that focused for wildlife conservation programme in Indian subcontinent owing to their uncompromised habit and habitats. Though the entomological tools has been using since ancient past for homicidal cases but it could not be applied ever for investigation of wildlife crime pertaining to evidence collection for prosecution and conviction of poachers. Most often carcass of free ranging tigers in the protected or unprotected forest areas being located under badly putrefied or decomposed stages. Thus identification of carcass needs alternative techniques instead of conventional pathological examinations to collect the facts behind the crime scene. During study period, carcasses of tiger of Kanha Tiger Reserve and Pench Tiger Reserve were examined carefully with the assistance of forest officers and veterinary physicians. The biological samples were collected for assessment of postmortem intervals by following standard protocols. The maggots (8-10) from individual carcass were dissected with the help of scalpel under stereoscopic microscope and teguments of the maggots were separated in sterilized container. From each sample DNA was extracted using DNeasy Blood and Tissue kit and amplified for the Cytochrome oxidase subunit I (COI) gene using commercially available specific primers. The PCR products were sequenced unidirectional and the sequence were identified using nucleotide BLAST by NCBI. The occurrence of *Chrysomya megacephala* (Fabricius, 1794) in both carcasses of tigers envisaged that blow flies of *Chrysomya* spp. attract earlier towards carcasses and lay eggs around orifices of the animal. The results emphasized the strength of forensic entomology not only for species identification of the blowflies but also useful for knowing the succession waves of forensically important insects in and around the protected forest areas.

Keywords: Maggots, blowflies, DNA, Tiger

Introduction

Biodiversity encompasses the multiplicity of genes, species and ecosystem that represents natural wealth of the living planet. Indiscriminate uses of non renewable resources, human made hazards and encroachment of forest boundaries are responsible for squeezing of wilderness in protected and non protected forest areas [1]. Retaliate killing of big cats and out breaks of deadly infectious diseases are alarming factors for extinction of threatened species of wild animals thus initiation of alternative techniques for forensic analysis is the need of hours to collect the evidence behind the crime scene for prosecution and conviction of culprits and poachers [2, 3]. Royal Bengal Tiger is the majestic species focused for wildlife conservation programme in India and adjoining countries owing to their uncompromised habit and habitats [4]. According to the perusal of available literature, application of forensic entomology was yet not applied ever in wildlife crime for collection of evidences behind the carcass encounters [5]. Blow fly maggots (Diptera: Calliphoridae) becomes one of the prominent biological tools to unfold the mystery behind the crime scene and plays important role for estimation of post mortem intervals, post mortem transfer or presence of drugs or poisons on the carcass [6]. Blow flies of Calliphoridae family also assist in decomposition process and are the earliest insects to attract towards corpse thus their presence may use for determination of elapse time of death mostly when carcass found in decomposed stages [7]. Morphological identification of blow flies is rather complicated owing to phenotypic similarities amongst sub-species. Hence, molecular characterization of forensically important blowflies' is precise, reliable and rapid for species identification of all developmental stages [8-10]. Limited efforts were made for wildlife forensic whilst entomological techniques has potentials to provide even the unexplained facts

behind the wild animal poaching or when decomposed carcasses come across in protected and non protected forest areas. Entomological techniques has been applied for the first time in calculation of elapse time of death from maggots recovered from two illegally killed black bear cubs in Manitoba, Canada and identified the blowfly species on the basis of maggot's morphology ^[11].

Therefore, the present study was focused on collection of maggots recovered from carcasses of free ranging tigers for identification of blowfly species occurrence on the basis of DNA characterization to utilize the technique in wildlife forensics.

Materials and Methods

Specimen collection. Maggots from an individual tiger carcass was collected with the help of forest officers and wildlife veterinary physician and divided into two parts, one part was kept in glass bottle containing 90% ethanol for gross morphological identification of maggots and their larval stages while another part was kept in amber bottle with silica gel and brought to the laboratory as early as possible for the further examination.

DNA extraction: Total DNA was extracted from maggot's using DNeasy Blood & Tissue kit from QIAGEN. The extracted DNA was eluted in 200µl of elution buffer and kept at -70°C for long term storage. During the study of blowfly maggots recovered from carcasses of free ranging tigers were analyzed by DNA characterization and a fragment of 229bp of COI gene was amplified.

PCR Amplification: Mitochondrial DNA was extracted and a 229 base pair nucleotide of the mitochondrial cytochrome oxidase subunit I was amplified. The primers used in the study were designed based on the description ^[13, 14]: C1-N-2800 (5'-CATTTCAAGYCTGTGTAAGCRTC-3') and C1-J-2495 (CAGCTACTTTATGAGCTTTAGG). The PCR products were separated electrophoretically on 1% agarose gel and visualized after Ethidium bromide staining (Fig1).

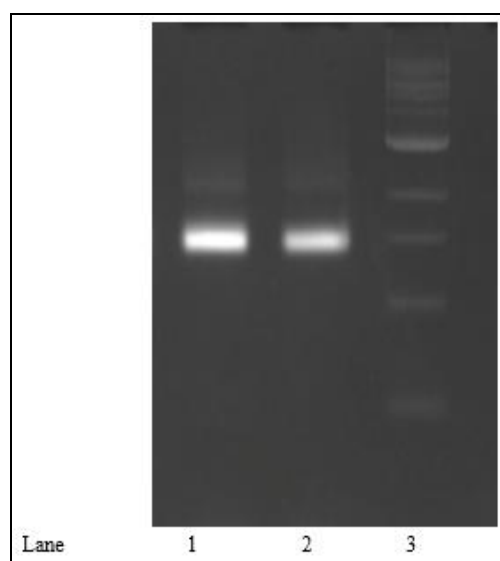


Fig showing the electrophoretic separation of the DNA. Lane 1 shows the DNA band at 229 bp of the maggots collected from the carcass of Tiger from Panna National Park. Lane 2 shows the DNA band at 229bp of the maggots collected from the carcass of Tiger from Pench Tiger Reserve. Lane 3 shows the Marker

DNA sequence alignment and phylogenetic analysis: The DNA sequences were subjected to nucleotide BLAST by NCBI. The reference sequences or previously reported blowflies were retrieved from GenBank and used for phylogenetic analysis namely *Chrysomya megacephala* (AF295551), *Chrysomya megacephala* (JN228996.1), *Chrysomya megacephala* (MK075815.1), *Chrysomya megacephala* (MK075813.1). Sequences were aligned and neighbor-joining trees were made using MEGA X software (Tamura Nei Model) ^[15]. All the sequence obtained was included in the phylogenetic analysis.

Results & Discussion

Sequencing and alignment of a total of 2 species was carried over 229 bp region and additional 4 sequences obtained from Genbank were compared. The sequences correspond to *Chrysomya megacephala* (Fabricius, 1794) a forensically important blowfly.

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-620.81) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site shown in (fig 2). This analysis involved 6 nucleotide sequences. There were a total of 323 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

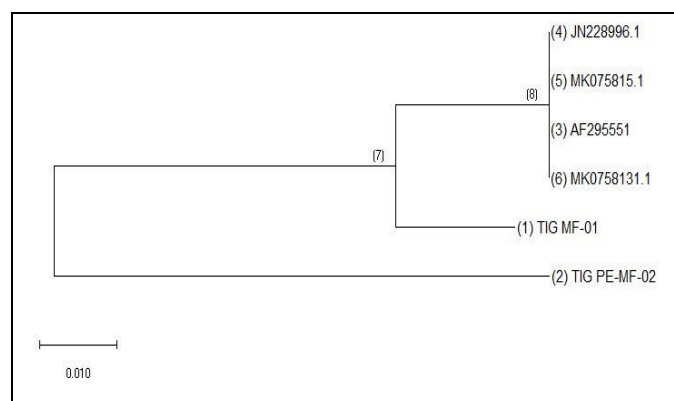


Fig 2: Evolutionary analysis by Maximum likelihood method.

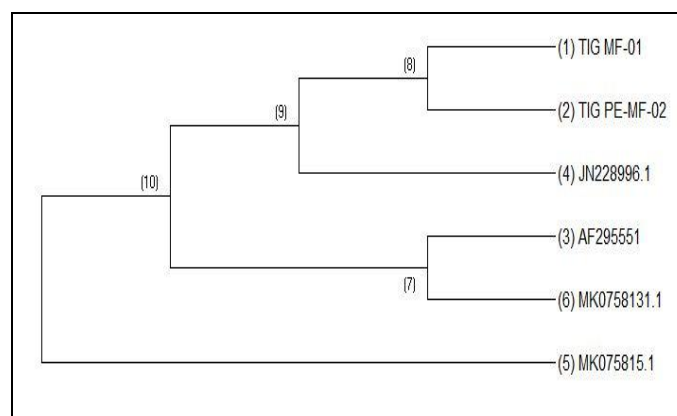


Fig 3: Maximum Parsimony analysis of taxa

Maximum Parsimony Analysis of Taxa

The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 8 most parsimonious trees (length = 43) is shown. The consistency index is 1.0 (1.0), the retention index is 1 (1.0), and the composite index is 1 (1.0) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Sub tree-Pruning-Regrafting (SPR) algorithm with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates) shown in (fig 3). This analysis involved 6 nucleotide sequences. There were a total of 323 positions in the final dataset.

Maximum Likelihood Estimate of Gamma Parameter for Site Rates

The estimated value of the shape parameter for the discrete Gamma Distribution is 200.0000. Substitution pattern and rates were estimated under the Tamura-Nei model (+G) [15]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G]). Mean evolutionary rates in these categories were 0.90, 0.96, 1.00, 1.04, 1.10 substitutions per site as shown in Table 1.

Table 1: Showing the maximum likelihood estimate of Gamma Parameter for Site Rates

TIG_MF-01					
TIG_PE-MF-02	0.124				
AF295551	0.035	0.125			
JN228996.1	0.035	0.125	0.000		
MK075815.1	0.035	0.125	0.000	0.000	
MK0758131.1	0.035	0.125	0.000	0.000	0.000

Maximum Likelihood Estimate of Substitution Matrix

Table 2: Showing the maximum likelihood estimate of substitution matrix.

	A	T/U	C	G
A	-	9.03	4.18	9.68
T/U	10.87	-	3.23	4.63
C	10.87	6.96	-	4.63
G	22.71	9.03	4.18	-

Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei model [15]. Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics* in Table 2. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100, the nucleotide frequencies are A = 37.84%, T/U = 31.44%, C = 14.58%, and G = 16.14%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -620.805. This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 323 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Blowfly succession on the carcasses of free ranging big cats is yet not studied and meager information is available. However, the credit goes to Anderson [11] who studied the blow fly infestation on the carcasses of two illegally killed black bear cubs in Manitoba, Canada and identified the blowfly species on the basis of maggot's morphology. Notwithstanding, the

molecular characterization of recovered maggots from tigers was not done so far as encountered in the present study. Plausibly, the big cats are directly linked with wildlife conservation and often victimizing for retaliate killing and poaching, henceforth the entomological tools may be the relevant alternative techniques to unfold the mystery behind the scene when carcass spotted in badly putrefied stage [12-14]. Subsequently, the postmortem interval change is directly proportional to the rate of colonization of blowflies on the carcass along with seasonal variations while morphological identification is crucial to undoubted identification [11]. Thus in the present study focused on DNA extraction and sequencing of maggots collected from free ranging tigers and yielded genotype and phylogenetic analysis has emphasized the occurrence of *Chrysomya megacephala* (Fabricius, 1794) in different Kanha and Pench Tiger Reserve of central India (Fig1). Similar findings also encountered in human cadavers during crime scene investigation in Malaysia [16]. However, the present work envisaged on identification of maggots that were collected from carcasses of free ranging Tigers would be useful for wildlife crime scene investigations particularly when poaching of tigers being noticed or suspected for alteration in the crime scene areas. Nonetheless, the role of blowflies in wildlife crime is essential part because mostly carcass found in liquefied or badly decomposed conditions, even some time only part of the carcass is available [14, 15]. In such circumstances, the conventional pathological method of investigation seems less potent to explain the cause of death or any suspected abuse occurred behind the scene [16-18]. Thus DNA characterization of maggots revealed the occurrence of blow flies species and their gut contents may also be used for species identification of carcass remains [19]. The attempts of the present study may helpful in collection of evidences for calculation of elapse time of death as well as evidences for prosecution and conviction of poachers as well.

Conclusion

The carcass examination of free ranging tigers in Kanha and Pench Tiger Reserve of Madhya Pradesh envisaged the blow fly species i.e. *Chrysomya megacephala* (Fabricius, 1794) that attracts earlier towards carcasses of big cats and lay eggs in orifices which develop later as maggots. The study appears land mark for use of entomological tools for collection of evidences during crime scene investigations particularly when carcass spotted in badly putrefied conditions. In such circumstances, the maggot on the corps helps not only to calculate the elapse time of death but also indicates about the evidences linked with wildlife crime as well.

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