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DNA characterization of maggots recovered from carcasses of wild animals

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Abstract
Identification of fly maggots recovered from carcasses help in estimation of elapse time of death as well as forensic related aspects. Proper identity of recovered maggots may only be confirmed by sequencing of the mitochondrial DNA analysis. During the study period, the blowflies maggots were recovered from different carcasses of free ranging wild animals including leopards (*Panthera pardus*), hard ground swamp deer (*Cervus duvaucelii branderi*) and langur (*Presbytis entellus*) etc. Different instar of maggots were collected in silica gel and brought to the laboratory and washed through sterile water. The maggots (6-8) from individual carcass of wild animal were collected and stored in -70 ^{0}C and then used for blowfly's species identification. 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 aligned using BLAST. Results envisaged that the protected areas of Madhya Pradesh including tiger reserves and national parks have 2 types of blowfly species i.e. *Chrysomya rufifacies* and *Hemipyrella ligurriens* that attract earlier towards carcasses and lay eggs in orifices of the wild animal's carcasses which develop later as maggots. The results are encouraging and helpful in strengthening of wildlife forensics.

Keywords: Blowfly, maggots, DNA, species identification

Introduction
Blow fly maggots (Diptera: Calliphoridae) became 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 [4]. Blow flies of Calliphoridae family also assist in decomposition process and are the earliest insects to infest a corpse thus used for determination of elapse time of death mostly when carcass found in putrefied conditions [9]. 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 [1, 6, 8, 14]. The application of forensic entomology is often in use for collecting evidences from homicidal crime scene since ancient past [5, 10, 11]. However, limited efforts were made for wildlife forensic whilst entomological techniques has potential to unfold the mystery behind the wild animal poaching or when decomposed carcasses come across in protected and non-protected forest areas. Entomological techniques was applied for 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 [2]. Therefore, the present study was focused on carcass examination and collection of maggots from free ranging carnivores and herbivores of different Tiger Reserves and National Parks of Madhya Pradesh. Simultaneously, identification of maggots of blowflies has been conducted on the basis of DNA characterization to utilize the techniques in wildlife forensics.

Materials and Methods
Specimen collection: Blowflies and third instars larvae (30-40 maggots) from the individual carcass of wild animals including leopards (*Panthera pardus*), hard ground swamp deer (*Cervus duvaucelii branderi*) and langur (*Presbytis entellus*) were collected and immediately divided into two parts, one part was kept in glass bottle containing 90% ethanol for gross Morphological identification while another part kept in amber bottle with silica gel and brought to the laboratory as early as possible and stored at deep freeze at -70 ºC till further analysis.

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 different free ranging wild animals 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 [11, 14] based on the description: C1-N-2800 (5'-CATTTCAAGYCTGTGTAAGCRTC-3') and C1-J-2495 (CAGCTACTTTATGAGCTTTAGG). The PCR mixture contained – 12.5µl of Mastermix for PCR amplification from Promega™, 9µl of molecular water, 0.5µl of each forward and reverse primers, 0.5µl of Bovine serum albumen (BSA) and 2µl of template DNA making the whole concentration to 25µl. Cycling conditions were 3 minutes 94 ^{0}C followed by 35 cycles of 94 ^{0}C for 1 minute, 45 ^{0}C for 1 minute and 72 ^{0}C for 1&half minute. A final extension period of 1 minute at 72 ^{0}C was used, which was followed by cooling at 4 ^{0}C. The PCR products were separated electrophoretic ally on 1% agarose gel and visualized after Ethidium bromide staining (Fig 1A&B).

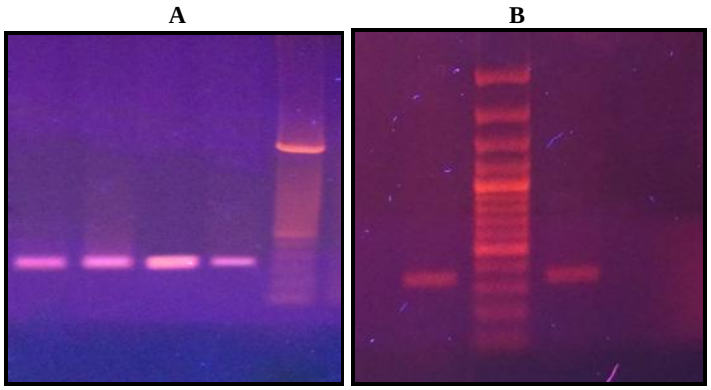


Fig 1 A: Mitochondrial COI showing DNA band on 229 bp of Blowflies Lane 1= *Hemipyrella lingurriens* 2= Marker and 3= *Chrysomya rufifacies* 1B: Mitochondrial COI showing DNA on 229 bp of Maggots recovered from Lane1=Swamp Deer (Barasingha), 2= Langur and 3&4 Leopards and 5= Marker Lane 1 2 3 Lane 1 2 3 4 5

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 Gen Bank and used for phylogenetic analysis namely *Chrysomya rufifacies* AH015276.2, *Chrysomya rufifacies* DQ328666.1, *Chrysomya rufifacies* DQ295070.1, *Chrysomya rufifacies* DQ098934.1 and *Hemipyrella ligurriens* AY842614.1, *Hemipyrella ligurriens* K037968.1, *Hemipyrella ligurriens* JX913759.1, *Hemipyrella ligurriens* JN228993.1. Sequences were aligned and neighbor- joining trees (Saitou and Nei 1987) were made using MEGA X software. All the sequence obtained was included in the phylogenetic analysis.

Results and Discussion

Sequencing and alignment of 2 species from the 4 carcasses of the wild animals was carried over 330bp region and additional 14 sequences from the Genbank to compare the data. The query sequences correspond to *Chrysomya rufifacies* and *Hemipyrella ligurriens*.

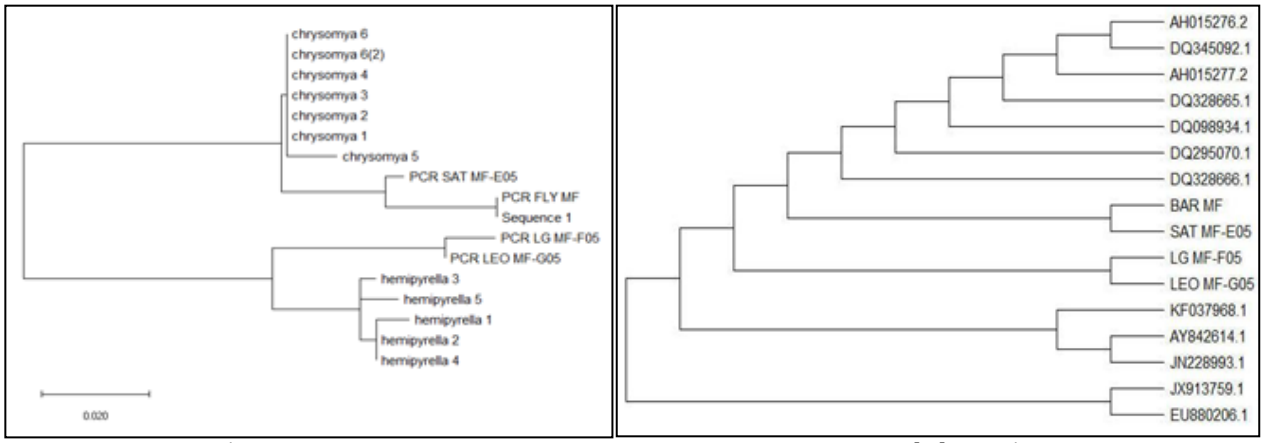
Identification

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-798.02) 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. This analysis involved 16 nucleotide sequences. There were a total of 330 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Fig 2).

Maximum likelihood method using gamma parameters

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-798.02) 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. This analysis involved 16 nucleotide sequences. There were a total of 330 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Fig 2).

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.3187. Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+G). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G]). Mean evolutionary rates in these categories were 0.00, 0.06, 0.28, 0.93, 3.72 substitutions per site. The nucleotide frequencies are A = 37.97%, T/U = 31.46%, C = 14.48%, and G = 16.09%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -791.103. This analysis involved 16 nucleotide sequences. There were a total of 330 positions in the final dataset. Evolutionary analyses were conducted in MEGA X for wild animal's *viz*. BAR_MF (Swamp Deer (Barasingha), LG_MF-F05 (Langur), LEO_MF-G05 (Leopard 1) and LEO SAT_MF-E05 (Leopard 2) (Fig 3).



Parsimony tree **Phylogenetic tree**

Fig 2: Showing Evolutionary relationship showing among the taxa inferred using MEGA X

BAR_MF															
LG_MF-F05	0.14														
AH015276.2	0.04	0.14													
DQ328666.1	0.04	0.14	0.00												
DQ295070.1	0.04	0.14	0.00	0.00											
DQ098934.1	0.04	0.14	0.00	0.00	0.00										
DQ328665.1	0.05	0.15	0.01	0.01	0.01	0.01									
AH015277.2	0.04	0.14	0.00	0.00	0.00	0.00	0.01								
LEO_MF-G05	0.14	0.01	0.13	0.13	0.13	0.13	0.14	0.13							
LEO SAT_MF-E05	0.02	0.14	0.02	0.02	0.02	0.02	0.03	0.02	0.13						
AY842614.1	0.16	0.06	0.12	0.12	0.12	0.12	0.13	0.12	0.05	0.14					
KF037968.1	0.15	0.06	0.12	0.11	0.11	0.12	0.12	0.12	0.05	0.14	0.01				
JX913759.1	0.15	0.06	0.11	0.11	0.11	0.11	0.12	0.11	0.05	0.13	0.01	0.01			
JN228993.1	0.15	0.06	0.12	0.11	0.11	0.12	0.12	0.12	0.05	0.14	0.01	0.00	0.01		
EU880206.1	0.14	0.03	0.12	0.12	0.12	0.12	0.12	0.12	0.03	0.13	0.01	0.01	0.01	0.01	
DQ345092.1	0.04	0.14	0.00	0.00	0.00	0.00	0.01	0.00	0.13	0.02	0.12	0.12	0.11	0.12	0.12

Fig 3: Pair wise distance of sequences using Maximum likelihood method by Tamura Nei Model

Estimates of evolutionary divergence between sequences
The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pair wise deletion option). There were a total of 330 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 1: Results from the Tajima's test for 3 Sequences (For *Chrysomya rufifacies*)

SN	Sequence	Configuration Counts
1	Identical sites in all three sequences	274
2	Divergent sites in all three sequences	01
3	Unique differences in Sequence A	04
4	Unique differences in Sequence B	34
5	Unique differences in Sequence C	04

NOTE. - The equality of evolutionary rate between sequences A (BAR MF) and B (LG MF-F05), with sequence C (AH015276.2) used as an out group in Tajima's relative rate test. The $\chi 2$ test statistic was 23.68 (P = 0.00000 with 1 degree[s] of freedom) P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages. This analysis involved 3 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option).There were a total of 317positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 2: Results from the Tajima's test for 3 Sequences (For *Hemipyrella ligurriens*)

SN	Sequence	Configuration Counts
1	Identical sites in all three sequences	271
2	Divergent sites in all three sequences	01
3	Unique differences in Sequence A	07
4	Unique differences in Sequence B	30
5	Unique differences in Sequence C	08

NOTE- The equality of evolutionary rate between sequences A (LEO MF-G05) and B (SAT MF-E05), with sequence C (AY842614.1) used as an out group in Tajima's relative rate test. The $\chi 2$ test statistic was 14.30 (P = 0.00016 with 1 degree[s] of freedom) P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages. This analysis involved 3 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 317 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.
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 correct identification [11]. Thus outcomes of the present study included forensic investigation and molecular characterization of collected maggots that breed directly on the carcasses. DNA extraction and sequencing of maggots collected from wild carnivores and herbivores yielded genotype and phylogenetic analysis emphasized the

occurrence of 2 species i.e. *Chrysomya rufifacies* and *Hemipyrella ligurriens* in different protected and unprotected forest areas of central India (Fig 1 A & B). Table.1 shows the uniform evolutionary divergence rate of *Chrysomya rufifacies*, similarly Table. 2 show the uniform evolutionary divergence rate of *Hemipyrella ligurriens.*

Even though forensic entomology applied since ancient past for medico legal cases while almost negligible efforts were made for veterolegal cases pertaining to wildlife crime. Anderson [2] initiated to identify the blowflies occurrence that were collected from the carcasses of two illegally killed black Bear cubs from Canada. Subsequently the application of forensic entomology is very common in homicidal cases where technology adopted for collection of evidences for prosecution and conviction of offenders thus molecular characterization techniques for identification of blowflies maggots and its gut contents may unfold the mystery behind the crime scene [3, 5, 7, 10]. The present work envisaged on identification of maggots and flies species (Fig 1A & 1B) that were collected from carcasses of free ranging Leopards, Swamp Deer (Barasingha) and Langur from different Tiger Reserves of Madhya Pradesh which may be the land mark for crime scene investigations particularly when animal poached and transferred to another areas. Simon [13] expressed that role of blowflies in wildlife crime is essential part because mostly carcass spotted in liquefied or badly decomposed conditions, even some time only part of the carcass is available. Thus DNA characterization of maggots revealed the occurrence of blow flies species on the carcass of wild animals may lead to calculation of elapse time of death and diversity of blowflies those attract first on the carcasses of wild animals. The attempts of the present study may helpful in collection of evidences for wildlife forensics.

Conclusion

The carcass examination of wild animals including carnivores and herbivores in different tiger reserves and national parks of Madhya Pradesh envisaged 2 types of blowfly species i.e. *Chrysomya rufifacies* and *Hemipyrella ligurriens* that attract earlier towards carcasses of wild animals and lay eggs in orifices which develop later as maggots. The study appears as an entomological tool for collection of evidences during crime scene investigations particularly when carcass spotted in badly putrefied conditions. In such circumstances, the maggot on the carcass 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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