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Molecular Phylogenetic study of larvae (*Chryosomya megacephela*) recovered from human corpse during crime scene investigation (Motinagar Thana suicidal case) at Sagar (India)

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ABSTRACT

DNA based identification of larvae sampled from Motinagar, Thana suicidal case of Sagar district, Madhya Pradesh was successfully demonstrated in the present study. It was found that the recovered larvae from the crime scene were identified morphologically as *Chrysomya rufifacies* but later molecularly confirmed to be *Chrysomya megacephala* by phylogenetic tree. Result from BLAST system revealed a 98-99% identity with *Chrysomya megacephala.* Phylogenetic analysis confirmed the presence of *Chrysomya megacephala* with 98-99% identity (match). The molecular study involved the sequencing of a total length of 1.3 kb encompassing the Cytochrome Oxidase I (COI) gene. This study will be very helpful to rule out the post mortem interval (PMI) due to correct species identification which can constrict the field of suspects and aid in the identification of the decedent in the near future also in India.

KEYWORDS: *Chryosomya megacephala,* mitochondrial DNA, COI, sequencing

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INTRODUCTION

Medico-legal entomological evidence plays diverse role as a forensic marker in criminal trials. In particular, determination of minimum postmortem interval (PMI_{min}) of a decomposed dead body, locations and sometimes in the manner of death, therefore, accurate species identification is necessary for exploring the full potential of insect evidence. Taxonomic identification has some limitations in the procedure of identification. Because of this, to ensure correct species identification, DNA based methods are now increasingly employed in the field of medico- legal entomology[1, 2]. Calliphoridae flies viz. *Chryosomya rufifacies* and *Chryosomya megacephala* are one of the first visitors on human corpse [3]. Now a day, mitochondrial DNA (mtDNA) is becoming a very useful tool in correct insect identification[4 5]. Mitochondial DNA (mtDNA) provides several advantages over nuclear DNA; therefore mtDNA becomes a better tool to determine closely related species for DNA based identification[6]. In India, there are many limitations to access human dead body at the crime scene, because forensic entomology is not very much popular in forensic practices. This study focus to examine the full potential of molecular identification of the first witness, that is fly and fly larvae recovered from human cadavers to compare the findings with the taxonomic identification of calliphoridae.

MATERIAL AND METHODS

(A) Insect collection and rearing

Insect larvae were collected with the help of soft nose forcep and kept into small plastic jar on 09.08.2016 at real crime scene (Temperature (24°C Avg.), relative humidity (97% Avg.) under Motinagar Police Station (0/16) Sagar. Maggots were reared (Temp 26.2°C) on an artificial diet using rearing method of Hung[7]. Emerge adult flies were used for taxonomic and molecular identification larvae were used for molecular identification (preserved in 90% ethanol).

(B) DNA extraction

Genomic DNA from whole fly and whole larvae were extracted using the DNeasy tissue kit (Qiagen, Germany) following the manufacturer's instructions. For fly and tissue samples, 180ul buffer ATL was used separately, added 20ul proteinase K in each vial, mixed by vortexing and incubated at 56°C until completely lysed. Added 200ul of buffer AL and mixed thoroughly by vortexing then added 200ul of ethanol (96-100%) and again mixed. The mixture was transferred into a DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. Flow was discarded and spin was placed in a new 2 ml collection tube followed by 500 ul buffer AWZ and centrifuged at 14000 rpm for 3 min. Flow was again discarded and spin was transferred

into a new 1.5 ml MCT and DNA was eluted by adding 200 ul buffer AE and incubated for 1 min at room temperature (20°C) and finally centrifuged at 8000 rpm for 1 min. Extracted DNA was checked electrophoretically on 1% agarose gel (Fig 1). The fraction of extracted DNA was spectrophoretically quantitated and diluted to 50ng/µL prior to PCR amplification step.

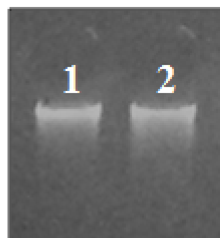

Figure 1: Genomic DNA Loaded in 1% Agarose Gel. Lane 1: Lavae genomic DNA. 2: adult fly (*Chrysomya megacephala*) genomic DNA.

(C) PCR amplification, sequencing and Phylogenetic analysis

Two opposing primers were selected for the PCR of the gene of Cytochrome Oxidase subunit 1 (COI) from published nucleotide sequences (Accession no. AY842621, AY842622, AY842623) and purchased from Chromous Biotech, Bangalore. The primers pairs composed of forward primer (Named C1-J-1751) of 5'GGAGCTCCTGACATAGCATTCCC3' and the reverse primer (Named UEA10) of 5'TCCAATGCACTAATCTGCCACATTA3' was designed to amplify a ~1.3 Kilo base pair of COI specific fragmented by PCR (Table 1). The PCR amplification mixture were prepared by containing the following components viz. 1X PCR reaction buffer, $MgCl_2$, dNTPs, template DNA (100 ng), Taq Polymerase and Forward & Reverse primers each. Amplification reactions were performed in a Thermal Cycler (Applied Biosystem). PCR cycling conditions were as follows: initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 sec, hybridization at 50°C for 5 sec and followed by elongation at 60°C for 4 min. It was found that optimal annealing/hybridization temperature was 50°C for COI region. The PCR product were separated electrophoretically on 1% agarose gel and visualized after ethidium bromide staining (Fig 2).

Table 1: Detail of PCR primers

S.No.	Location	Sequence	Reference
1.	CJ-J-1751	5'GGAGCTCCTGACATAGCATTCCC3'	8
2.	UEA10	5'TCCAATGCACTAATCTGCCACATTA3'	9

DNA sequencing was carried out using an ABI 3500XL Genetic Analyser Big Dye Terminator Version 3.1' cycle sequencing kit, POP_7 polymer & 50 cm Capillary array as polymer and capillary array, BDTv3-KB-Denovo_v 5.2 as analysis protocol and Seq Scape_ v 5.2 as data analysis software. The sequencing mixture containing the following components viz. Big Dye Terminator Ready Reaction Mix (4µL), Template (100ng/ul), Primer (10pmol/λ), Milli Q Water (3µL). The sequencing was determined for both forward and reversed strands. Sequence alignment

and phylogenetic tree by Maximum Likelihood Method were made using MEGA 7, bootstrap support derived from 5000 replicates. Aligned sequence of COI of *Chrysomya megacephala* after sequencing is shown in fig 3. The reference sequences of previously reported work on *Chrysomya megacephala* identification are shown in Table 2.

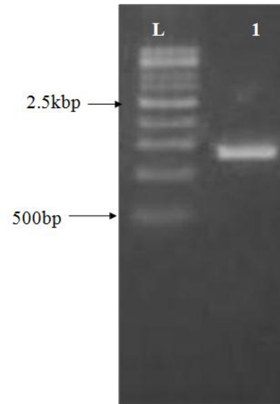

Figure 2: PCR Product loaded on 1% Agarose Gel. Lane description: L: 500 bp ladder, 1: *Chrysomya megacephala* (~1.3kb)

CCGTAGTCATATAACTTTATTATTAGTAAGTAGTATAGTAGAAAATGGGGCTGGAACAGGATGAACTGTTTACCCACCT
TTATCTTCTAATATTGCTCATGGAGGAGCATCAGTTGATTTAGCTATTTTCTCTTTACACTTAGCAGGAATTTCTTCAATT
TTAGGAGCTGTAAATTTTATTACAACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACCTTTATTTGT
ATGATCTGTAGTTATTACTGCTCTATTATTATTATTATCTTTACCAGTATTAGCTGGAGCTATTACTATATTATTAACTGA
CCGAAATCTAAATACTTCATTCTTTGACCCAGCAGGAGGAGGAGATCCTATTTTATACCAACATTTATTTTGATTCTTTG
GACATCCTGAAGTTTATATTTTAATTTTACCTGGATTCGGAATAATTTCTCATATTATTAGTCAAGAATCAGGAAAGAAG
GAAACTTTCGGATCTTTAGGAATGATTTATGCTATACTAGCTATTGGTCTATTAGGATTTATTGTATGAGCTCACCACAT
GTTTACTGTTGGAATAGACGTAGACACACGAGCTTATTTCACTTCAGCTACATAATTATTGCTGTACCAACTGGAATTAA
GATTTTCAGTTGATTAGCAACTCTTTACGGAACACAATTAAATTATTCTCCAGCTACTTTATGAGCTTTAGGATTTGTATT
TTTATTTACTGTAGGAGGATTAACTGGAGTTGTTTTAGCTAATTCATCATTTGACATTATTTTACATGATACATATATGTA
GTAGCTCACTTCCATTATGTTCTATCATGGGAGCTGTATTTGCTATTATAGCAGGATTTGTTCATTGATTCCCTCTATTTA
CTGGATTAACTTTAAATAGCAAGTTATTAAAGAGTCAATTTGCTATTATATTTATCGGAGTAAATTTAACATTCTTCCCT
CAACATTTCTTAGGATTAGCAGGTATACCTCGACGATACTCAGACTATCCAGACGCTTACACAGCTTGAAATGTAATTT
CTACAATTGGTTCAACAATTTCATTATTAGGAATTTTATTCTTCTTTTTCATTATTTGAGAAAGTTTAGTATCTCAACGAC
GAGTTTTATTCCCTGTTCCAAATAAATTCTATCTAATTGAATGATTAACTAA

Figure 3: Aligned sequence of *Chrysomya megacephala* (~1.3kb)

Table 2: Detail of NCBI GeneBank accessions (mtCOI) analysed

Species	Accession number	Locality	Reference
C. megacephala	AF295551	USA	10
C. megacephala	AB112830	Australia	11
C. megacephala	AB112841	Australia	11
C. megacephala	AB112846	Australia	11
C. megacephala	AB112847	Australia	11
C. megacephala	AB112848	Australia	11
C. megacephala	AB112861	Australia	11
C. megacephala	AY092761	Taiwan	12
C. megacephala	AY842619	Australia	13
C. megacephala	AY909053	Malaysia	14
C. megacephala	FJ153258	Thailand	15
C. megacephala	FJ614818	China	16
C. megacephala	FJ614817	China	16
C. megacephala	FJ614816	China	16
C. megacephala	GQ912669	Pakistan	16
C. megacephala	KC249623	China	17
C. megacephala	KC249624	China	17
C. megacephala	KC249625	Egypt	17
C. megacephala	KC249626	Egypt	17
C. megacephala	KC249673	China	17
C. megacephala	KC249674	China	17
C. megacephala	KC249675	Egypt	17

Table 3: Detail of specimens sequenced

Species	Location	Police station	Date of collection	Methods and duration of preservation
C. *megacephala*	Sagar (India) (23°5'N, 78°5'E)	Motinagar (0/16)	09 Aug 2016	F, More than 10 month

Note: F, Frozen

RESULT AND DISCUSSION

In this study, a suicide case of Motinagar, Thana of Sagar district, Madhya Pradesh was investigated (Fig 4). A case where the body was recovered from the closed room and declared as a suicide case after the investigation, during investigation of crime scene, entomological evidence as larvae were collected from the body as well as from the crime scene and the habitat was urban site, indoor with cloths on the body. The molecular identification of mitochondrial DNA (mtDNA) region was studied encompassing the Cytochrome oxidase subunit 1 gene (COI) gene. In this study, the complete 1.3 Kb nucleotide fragment was amplified (fig 3) and the phylogenetic analysis of collected larvae is shown in Fig 5. The detail of location, police station, date of collection and method & duration of preservation of *Chrysomya megacephala* are presented in table 3. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [18]. The bootstrap consensus tree inferred from 5000 replicates [19] is taken to represent the evolutionary history of the taxa analyzed [19]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated

taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches [19]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 100 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 536 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20].

At motinagar thana case study, the recovered larvae were identified morphologically as *Chrysomya rufifacies* but later molecularly confirmed to be *Chrysomya megacephala* by phylogenetic tree. Result from BLAST system revealed a 98-99% identity with *Chrysomya megacephala.* Phylogenetic analysis confirmed the presence of *Chrysomya megacephala* with 98-99% identity (match). Out of 100 subject sequences, 95 sequences were of *Chrysomya megacephala* only and rest 5 other *Chrysomya* sp. sequences viz. *Chrysomya putoria* (98% identity), *Chrysomya cabrerai* (98% identity), *Chrysomya pacifica* (98% identity), *Chrysomya saffranea* (98% identity) and *Chrysomya chain* (98% identity) was also found based on phylogenetic tree analysis.

Hence it was found that the larvae recovered from human corpse during crime scene investigation were of *Chrysomya megacephala* larvae and it was also evident that the blow (Calliphoridae) flies viz. *Chryosomya rufifacies* and *Chryosomya megacephala* were first to visit on human corpse [3]. Forensic insect analysis during crime case provides valuable information by calculating Post mortem Interval (PMI) of larvae and insects found on human dead body and narrowing the investigation process.

Motinagar thana case -indoor with cloths

Figure 4: Dead body recovered from crime scene investigation (Motinagar, Thana case) in Sagar, M.P

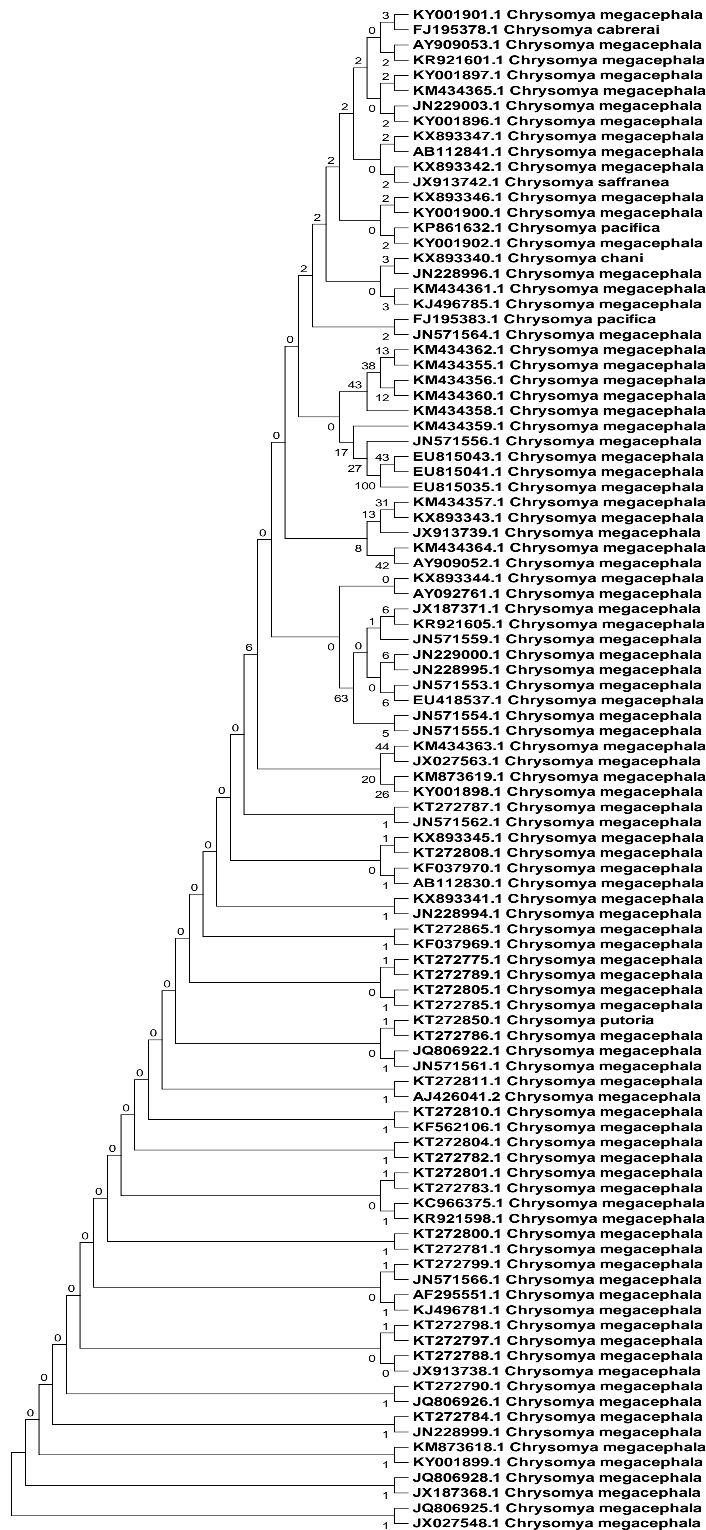

Figure 5: Maximum Likelihood tree illustrating the presence of *Chrysomya megacephala* and phylogenetic relationships among blow flies (family: Calliphoridae) recovered from crime scene investigations based on COI gene of 1.3 Kb

CONCLUSION

The current study successfully identified the collected larvae during crime scene investigation of Motinagar Thana case at Sagar, M.P. The entomological evidence collected was identified as

Chrysomya megacephala by DNA based analysis. This will further prove valuable in estimating the PMI in forensic investigation.

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INTEREST OF CONFLICT

Author declares no conflict of interest.

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