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Identification of Forensically Important Insect *Chrysomya megacephala* (Diptera: Calliphoridae) in Criminal Investigation from Nagpur Region

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Abstract: *Chrysomya megacephala* (Diptera: Calliphoridae) also known as oriental latrine fly is blow fly species widely distributed in many parts of the world. This fly has forensic importance as it infests on carrion soon after death and helps not only to estimate the post-mortem interval (PMI) but also to detect poisoning in a putrefying body. The present study was carried out on morphological identification of *Chrysomya megacephala* using key Lutz, 2018 and Cytochrome C Oxidase (COI). The special attention was focused on the tertiary morphological characteristics of flies collected from Nagpur region. The new COI subunit I (588 bp) gene sequence of *Chrysomya megacephala* have been added to GenBank (Accession No.- MN082633).

Keywords: Blowfly, Calliphoridae, *Chrysomya megacephala*, Cytochrome C oxidase (COI) gene, MN082633, Molecular, Morphological identification.

I. Introduction

Necrophagous forensic insects are attracted by decaying corpses and colonize on the body immediately after death. Therefore, they help in solving the problems related to mysteries of a crime like murder, suicide, sexual molestation, wildlife crimes and other legal issues. They provide the information on time of death, child-neglect and abuse, chemical intoxication, the path followed by the culprit, the place of death, etc. As fly specimens found in forensic investigations generally have a similar appearance, the accurate identification of forensically important insects, such as flies were a crucial step for them to be used as evidence in criminal investigations. The incorrect species identification will invalidate the estimated post mortem interval and impact other interpretations of the evidence [1].

The Calliphoridae is a large group in the Diptera, and 1,000 species in 150 genera are recognized worldwide [2]. The Calliphoridae are probably most important not only because they occur in abundant numbers but also they are one of the earliest groups to find the corpses [3] and classified as follows: Kingdom - Metazoa, Phylum - Arthropoda, Class - Hexapoda, Order - Diptera, Suborder - Brachycera and Family - Calliphoridae. This family includes the subfamily Calliphorinae, Chrysomyinae, Lucilinae, Polleniinae, Melanomyinae, Auchmeromyiinae, Ameniinae, Rhiniinae, etc. They are characterized typically as being metallic blue, green, purple, or non-metallic in colour and they vary in size [4].

Morphological study is most common method to identify adult insects. The morphological identification of adult flies can be carried out by using 3 step classification system which includes various specific characters (identification marks) which is used for the identification of Calliphoridae species.

- Primary classification (For identification of family),
- Secondary classification (For identification of subfamilies of Calliphoridae) and
- Tertiary classification (For identification of genus and species of Calliphoridae).

The genus level classification of Calliphoridae is primarily based on external adult characters [5], whereas identification at the species level is otherwise highly dependent on the morphology of the male genitalia, especially the aedeagus [6].

The main aim of present study was to carry out morphological and molecular identification of blow fly species form Nagpur region for the first time. The new COI gene sequences have been added to GenBank, so that it will be helpful to keen researchers for correct identification of blow fly species for future research.

II. Material and Methods

Collection: The Adults flies were collected from meat shops of Nagpur city using insect collecting net and were preserved in collecting vials containing 70% ethanol. Before addition of 70% ethanol [7], one-two legs of flies were removed and stored in separate tube for the extraction of DNA.

A. Morphological Identification

Morphological identification of forensically important adult blowflies is challenging job. The morphological identification of collected flies were carried out in research laboratory under 'Stemi 305 ZEISS' stereomicroscope. A good quality images of blowflies were taken by using advanced 'Axiocam 105 colour' camera associated with 'Zen 2.3 Lite' software. The morphological identification of adult flies were done by using 3 step classification system which includes various specific characters (identification marks). The genus and species level classification was carried out in the tertiary classification which is primarily based on external adult characters of Calliphoridae. The anterior thoracic spiracle and the lower calypter are important in the morphological differentiation of this species from its close relatives [8].

The tertiary classification of Calliphoridae were carried out using specific diagnostic morphological keys given by Lutz, 2018 [9].

B. Identification Keys for Chrysomya Species (Lutz, 2018)

- Stem vein of wing without row of hairs on dorsal surface, greater ampulla without stiff, erect hairs.

  Stem vein of wing with row of hairs on dorsal surface, greater ampulla with stiff, erect hairs .... 2.
- Anterior spiracle blackish brown .... 3.

  – Anterior spiracle white or yellowish .... 4.
- Eyes of male touching and with sharply demarcated and strikingly large upper facets; frons of female with fronto-orbital plate dark; lower calypter brownish with dark rim .... *Chrysomya megacephala* (Fabricius, 1794).

  – Eyes of male close but not touching and without demarcation between dorsal and ventral facets; frons of female with fronto-orbital plate reddish; lower calypter uniformly white ....*Chrysomya bezziana* (Villeneuve, 1914).
- Anterior wing veins and membrane black or strongly infuscated …………………5.

  – Anterior wing margin hyaline; if weakly darkened (some specimens of *Chrysomya putoria*), then ground colour of face and genal dilation dark .... 7.
- Eyes of male with sharply demarcated and strikingly large upper facets; fronto-orbital plate of female totally orange.. *Chrysomya marginalis* (Wiedemann, 1830).

  – Eyes of male without demarcation between dorsal and ventral facets; fronto-orbital plate in female dark at least in upper part...6.
- Eyes of male widely separated, frons at its narrowest point measuring 0.5 or more times eye width; frons of female subparallel, fronto-orbital plate with upper part black and lower part bright, reddish... *Chrysomya laxifrons* (Villeneuve, 1914).

  – Eyes of male touching; frons of female conspicuously narrowed on lower half, fronto-orbital plate entirely black ....*Chrysomya inclinata* (Walker, 1861).
- Dorsal surface of thorax shining green with little, evenly distributed dusting; gena reddish-yellowish in ground colour; lower calypter white; outer vertical seta of male well-developed ....*Chrysomya albiceps* (Wiedemann, 1819).

  – Dorsal surface of thorax with conspicuous whitish dusting, forming characteristic patterns of dusted and undusted surfaces; genadarking round colour; lower calypterdarker; outer vertical seta of male absent....................................8.
- Pre-sutural area of thorax with conspicuous black 'L' shaped marking on each side of midline, strongly whitedusted median vitta proceeding forwards from suture to front of thorax; abdomen with last two segments shiny brassy/green and contrasting strongly with bluish anterior segments .... *Chrysomya chloropyga* (Wiedemann, 1818).

  – Presutural area of thorax without conspicuous paired L shaped markings, but with vague lateral dark spots, white-dusted median vitta extending forwards hardly halfway to front of thorax; abdomen with last two segments concolourous with green/bluish anterior segments .... *Chrysomya putoria* (Wiedemann, 1830).

C. Molecular Identification

DNA barcoding, using COI, has demonstrated for rapid and accurate identification of Calliphoridae at species level. For molecular identification of unknown fly, preserved legs of fly were removed under sterile condition and kept in Eppendorf tube containing 3 ml of fresh absolute alcohol.

D. DNA Isolation

DNA from fly were extracted by using the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd. The fly sample was grind into a powder form and mixed with 700 µl of lysis buffer and 4 µl of RNAse in a 2 ml micro centrifuge tube. After the DNA extraction**,** DNA concentration was measured by Qubit 3.0.

E. Amplification and Purification

In the Polymerase Chain Reaction (PCR), the COI gene fragment was amplified using 2 Universal primers:

Primer Name Sequence Details Number of Base

HCO2198 5’TAAACTTCAGGGTGACCAAAAAATCA3’26

LCO1490 5’GGTCAACAAATCATAAAGATATTGG3’25

5 µL of isolated DNA was added in 25 µL of PCR solution. PCR was performed with initial denaturation at 95 °C so as to break the DNA strands to single stand. Repeated amplification cycles were performed at 95 °C for 45 secs, 40 °C for 60 secs, and 72 °C for 60 secs with a final Extension at 72 °C for 10 min. In this process amplified specific DNA fragments were harvested. The unincorporated PCR primers and dNTPs were removed from the PCR products by using Montage PCR Clean up kit (Millipore). Sequencing reactions were performed using an ABI PRISM BigDye^{TM} Terminator Cycle Sequencing Kits with the LCO1490/HCO2198 primers and AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

F. DNA Sequencing

Single-pass sequencing protocol was performed on each template using LCO1490/HCO2198 universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water, subjected to electrophoresis and sequencing were performed by using ABI 3730xl sequencer (Applied Biosystems).

G. BLAST Analysis

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of the sequence with the closely related sequence of blast was performed, followed by multiple sequence alignment. The gene sequences obtained from the sequencer was converted into the FASTA format and used for the pair wise alignment. BLAST analysis is used from a web link http://blast.ncbi.nlm.nih.gov/. The BLASTN program accepted the FASTA format of the sequence. Homology for the given sequence was analysed that helped in identification of species. The algorithm parameters were set as the E-value (expect value) at 10, match/mismatch score as 1/-2, gap cost as linear and BLASTN set as mega BLAST using highly similar sequences in the query. These sequences were retrieved in FASTA format and used to build up the phylogram in the CLUSTALW and MEGA5 software.

H. Multiple Sequence Alignment

Obtain gene sequence was used to perform the multiple sequence alignment by CLUSTALW program. CLUSTALW performs multiple sequence alignment by accepting the closely linked sequences in the FASTA format file using web http://www.ebi.ac.uk/Tools/msa/clustalw2/. During alignment, 10 best scored aligned sequences were generated in the BLAST analysis. These sequences were downloaded in FASTA format and used in the analysis. In a ClustalW program, alignment parameters were set as follows: DNA weight matrix set as IUB, Gap penalty as 10, Gap Extension as 0.20, Gap distance as 5 and clustering method as Neighbour joining. In an output result, the file was retrieved as. DND which was used to build the phylogram in MEGA5 software based on the alignment obtained in CLUSTALW analysis.

I. Phylogenetic Analysis

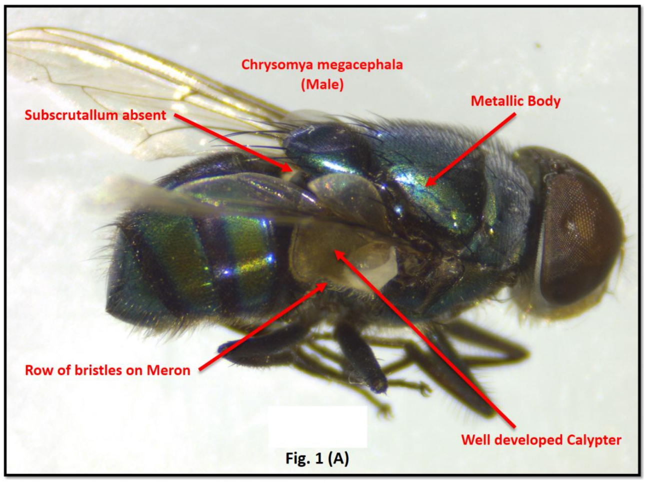
For the phylogenetics analysis, DND file obtained from CLUSTAL alignment was used for the phylogram built up by using the MEGA5 software.

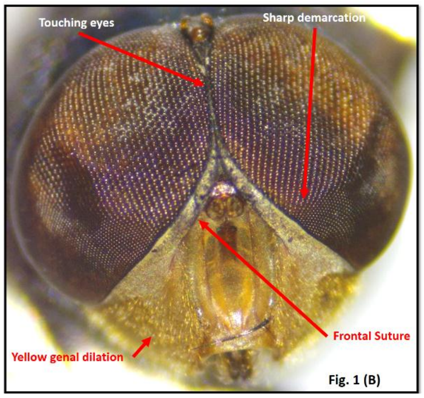
III. Results

This is a prime study on morphological and molecular identification of forensically important Blow fly from Nagpur region. Morphological identification of collected flies were carried out using 3 step classification system under microscope using the morphological key given by Lutz, 2018 and the DNA sequence data of cytochrome oxidase subunit I (COI) gene.

In Primary Classification, it is observed that the collected adult fly has all the characters related to the Calliphoridae family [Fig. 1 (A), (B) and (C)]. It has shiny metallic blue, green colouring of thorax and abdomen and well developed calypters. It has three-segmented antennae and hairy aristae and Frontal suture was clearly visible. The postscutellum was absent in the fly and have bristles on their meron and also have branched Rs veins [10], [11]. Therefore, the collected blow fly belongs to the Calliphoridae family.

Secondary Classification of collected blow fly was specified to subfamily characteristics. During the Secondary Classification, it is observed that the fly has metallic body [Fig. 1 (A)] with yellow coloured genal dilation [Fig. 1 (B)]. Lower calypter covered with dense setae or hair [Fig. 2] and Stem-vein have hairs on dorsal side [Fig. 3] which shows that this fly belongs to Subfamily Chryosomyinae [10], [11]. As it is observed that collected fly has the yellow genal dilation, therefore, there is need to search for those Chryosomyinae flies who have yellow genal dilation.





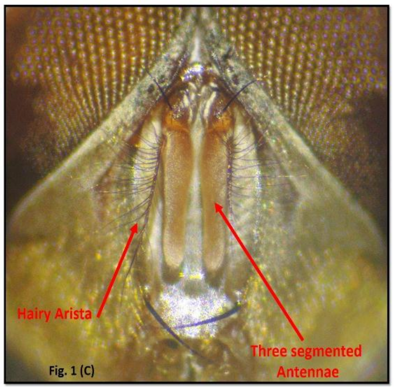


Fig. 1: Primary Characteristics of Collected Fly

During the tertiary classification, it is observed that collected Calliphoridae fly has hairs on dorsal surface of Stem vein [Fig. 3], greater ampulla with stiff, erect hairs, touching eyes with sharp demarcation between lower (small) and upper facets (large) [Fig. 1 (B)], brownish lower calypter with dark rim [Fig. 2], which shows that this fly belongs to the species *Chrysomya megacephala* (Lutz, 2018).

For the molecular identification, single-pass sequencing protocol was successfully performed on each template of 588 bp fragment of the extracted mitochondrial COI gene, using LCO1490/ HCO2198 universal primers. The sequence obtained was identified correctly in the BLAST search tool. The phylogenetic analysis of the flies with the various closely related species was carried out using MEGA5 software followed by multiple sequence alignment. The DND file obtained from CLUSTAL alignment was used for building the phylogram which shows close homology with *Chrysomya megacephala* [Fig. 4].

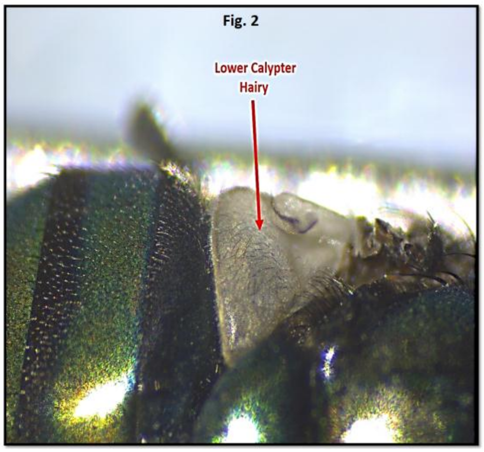


Fig. 2: Hairy Lower Calypter

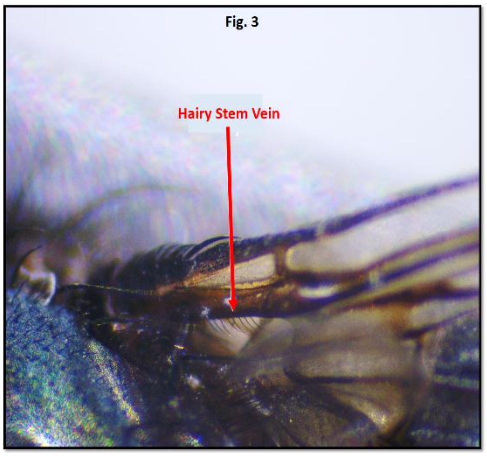


Fig. 3: Hairy Stem Vein

DP4 gene sequences have the highest similarity sequence with *Chrysomya megacephala* SCAU-DE-LD-C26 [KY020 769].

The estimated sequence of *Chrysomya megacephala* is deposited in NCBI gene bank (http://www.ncbi.nlm.nih.gov) under the Title: identification of forensically important insects (Diptera-Calliphoridae) in criminal investigation from Nagpur region and the GenBank accession Number of the samples was obtained (MN082633.1).

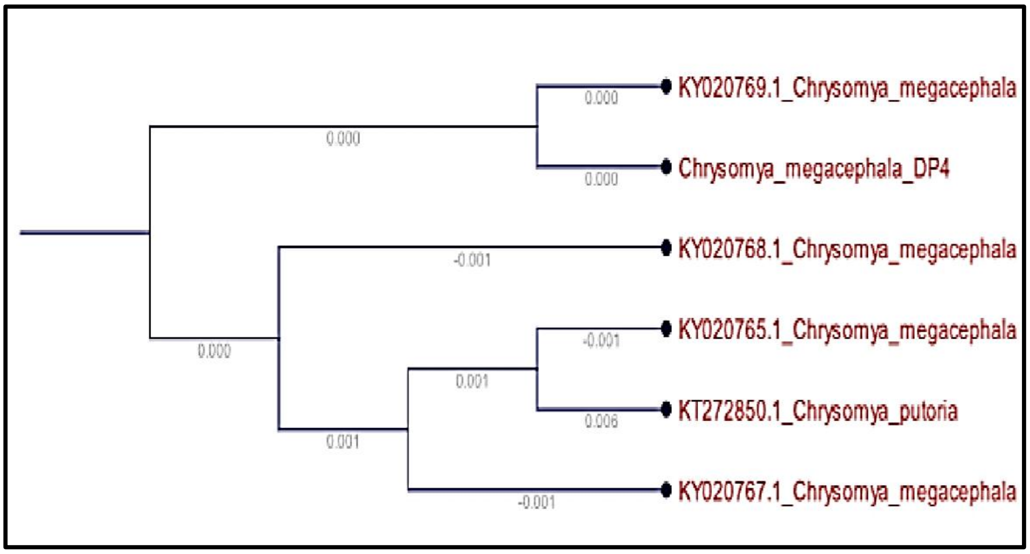


Fig. 4: Phylogenetic Tree of the Suspected Blowfly Sample with the Distance Between the Closely Related Species

IV. Discussion

This is first and unique study to unravel the identity of unknown blow fly species, collected from the Nagpur region, Maharashtra, using morphological identification key and COI gene analysis.

Fahd and Zambare, 2017 [12] were carried out Molecular identification of forensically important blowflies (Diptera: Calliphoridae). They described the utility of Cytochrome C Oxidase subunit I gene (COI) DNA barcode for the identification of forensically important blowflies to confirm the morphological identification.

Sathe *et al*., 2013 [13], Jadav, 2014 [14] and Bharamal, 2016 [15] also reported various species of blow flies (Calliphoridae) mainly from the Western side of Maharashtra.

Bharti *et al.*, 2017 [16] also carried out the DNA-Based Identification of Forensically Important Blow Flies (Diptera: Calliphoridae) from India and develop cytochrome oxidase subunit 1 (COI) reference data from nine species (belonging to three subfamilies namely Calliphorinae, Luciliinae and Chrysomyinae) of blow flies.

Wei-Yun Chen *et al.*, 2004 [17] carried out molecular identification of forensically important blow fly species (Diptera: Calliphoridae) in Taiwan and constructed species-specific molecular primer key for rapid and accurate identification of species. The above references support the present morphological and molecular homology with *Chrysomya megacephala.*

V. Conclusion

Using morphological i.e., primary, secondary and tertiary classification and molecular identification, collected forensically important blowfly is found to be *Chrysomya megacephala.* More study is needed from Nagpur Region for unravelling the various forensically important species for its use in investigation.

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