



## MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF FORENSICALLY IMPORTANT BLOW FLY *CHRYSOMYA MEGACEPHALA* (F.)

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### ABSTRACT

Considering the significance of *Chrysomya megacephala* (F.) (Diptera: Calliphoridae) and their larval instars in forensic-entomological investigations, a culture of *C. megacephala* was established and morphological features of second and third larval instars were studied under a compound microscope. To validate the species' morphological identification, sequencing of the 'standard barcode region' (658 base pairs of mitochondrial cytochrome oxidase subunit I gene) was performed and this sequence was subjected to BLAST analysis using the NCBI database which acknowledged it as *Chrysomya megacephala* with 99.39% identity with sequence of same species collected from Maharashtra, India. The phylogenetic analysis congregated Jaipur population separately than other samples from different regions of India, however, not much variations were recorded. This is the maiden report of molecular identification of *C. megacephala* from Rajasthan.

**Key words:** *Chrysomya megacephala*, Diptera, morphology, adult, maggots, DNA barcoding, BLAST, ClustalW, phylogenetic analysis

Forensic entomology is an enabling science that assists the investigations at any crime scene. *Chrysomya* being cosmopolitan are the pioneer colonizers on decaying human bodies, thus, act as crucial evidence during the forensic analysis of a crime investigation, especially in estimating the post-mortem interval (PMI) (Amendt et al., 2004). It is imperative to identify the exact species during estimation of PMI, but classical morphology-based methods are generally strenuous (Wallman, 2001). Therefore, there has been a consistent growth in DNA-based techniques for molecular species identification since the first positive endeavour to comprehend important forensic insect species by Sperling et al. (1994). As insect specimens collected from corpses are mostly immature developmental stages which are imperceptible externally making them morphologically inappropriate for forensic exploration (Catts and Goff, 1992), therefore, DNA based methods for their identification are propitious. Hence, the present study aimed at observing the morphology of the immature stages of *C. megacephala* to provide distinctive features. The identification of *C. megacephala* was confirmed by DNA barcoding.

### MATERIALS AND METHODS

A random clutch of eggs was collected from one of

the meat baited fly traps set for studying abundance of carrion flies in the campus of University of Rajasthan, Jaipur with latitude 26°1'36" North and longitude 75°4'32" East. The clutch of eggs was transferred on minced meat in a plastic glass, covered with fine mesh cloth and placed inside a cage for further development under natural environmental conditions. The adult flies so emerged were provided with milk powder, sugar, pork liver/flesh as food (a protein source for sexual maturation and oviposition) and water ad libitum (Day, 2006). The adult specimens were identified as *Chrysomya megacephala* using morphological keys (Carvalho and Mello-Patiu, 2008; Szpila, 2012).

For morphological observations of 2<sup>nd</sup> and 3<sup>rd</sup> instar larvae the hydroxide clearing method (Sukontason et al., 2004) was used. Larvae (both instars) were placed in a test tube containing hot water (80° C) to restrain them from dwindling in size (Adams and Hall, 2003). The dead larvae were preserved in 70% alcohol and later two incisions were made. An anterior incision was put through the second thoracic segment to observe the cephalopharyngeal skeleton and anterior spiracle. Another, posterior incision was made through the 11<sup>th</sup> segment for viewing the physiognomies of the posterior spiracle. These were cleared off using 10% potassium hydroxide solution and later neutralized

using a solution of 35% ethanol and 1% glacial acetic acid. Subsequently, the specimens were dehydrated and mounted for observing morphological characteristics under a compound microscope (Bunchu et al., 2012). To investigate the morphology of pupa, the anterior and caudal parts of puparia were cleared using 20% potassium hydroxide solution (Sukontason et al., 2007). Same procedure was repeated carefully for sample processing and the significant features of anterior and posterior sections were recorded along with photographic evidence.

For molecular identification, the genomic DNA was extracted from a single individual fly using Nucleospin Tissue XS Kit (MN), following the manufacturer's protocols. Mitochondrial cytochrome oxidase I (mtCOI) gene region from total DNA was PCR amplified using specific primers set (LepF -attcaaccaatcataagatattgg and LepR-taaactctggatgtccaaaaaatca) (Hajibabaei et al., 2006; Jindal et al., 2017). The amplicon was gel purified using 'QIA quick gel extraction kit' Qiagen and was cloned using pTZ57R/T vector using 'InsT/A Clone PCR product cloning kit' (Fermentas Life Sciences) as per the instructions set by the manufactures. The nucleotide sequence of recombinant clones was determined through custom sequencing services of M/S Xcelris (Ahmedabad, India). The sequences were edited and subjected to BLAST analysis in the NCBI database for sequence homology. The nucleotide sequence once analyzed and identified was submitted to the GenBank database for future references. The nucleotide sequences of blowfly collected from India and other countries (one each) were downloaded from NCBI GenBank and multiple alignment is done with ClustalW programme. The phylogenetic tree was created using maximum likelihood statistical method with the MEGA7 program using Tamura 3-parameter model.

## RESULTS AND DISCUSSION

The hydroxide clearing technique used in the present investigation, allowed easy examination of the internal sclerotized structures. Smith (1986) also used 10% KOH to clear the cuticle of larvae of 'true flies' whereas, Wirth and Marston (1968) and Turner (1990) cleared cephalopharyngeal skeleton of calliphorid larva in saturated phenol/absolute ethanol solution. Likewise, Smith (1986) who emphasized on characteristic features like anterior and posterior spiracles and cephalopharyngeal skeleton for identification of larvae up to species level, the larval instars of *C. megacephala* were studied for their anterior and posterior spiracles and cephalopharyngeal skeleton in the present study.

Larval instars of *C. megacephala* displayed distinctive muscoid shape, having 12 segments, which were pointed at anterior and truncate at posterior end. A cephalopharyngeal skeleton which comprises of mandibles and sclerites and mouth hooks was present at anterior end. Anterior spiracle was observed between second and third segments having 10-12 spiracular branches and orifices (Fig.1). On the posterior end, two brown circular areas were present on the horizontal face of the terminal segment, these were posterior spiracles (Fig. 2). The margin of the caudal segment of the larva was surrounded by projections, known as tubercles. The specific larval stages (LI, LII, LIII) were identified by the number of slits present in posterior spiracle. The cephalopharyngeal skeleton (Fig. 3) in second instar was almost complete and the posterior spiracle was having two separate spiracular slits with light pigmentation over an incomplete peritreme (Fig. 4). The spines present on the third segment of the larvae exhibited rows of single-pointed tips. The third instar larvae were the largest with robust cephalopharyngeal skeleton (Fig. 5). It was comprised of longer dorsal cornua than ventral cornua. The posterior spiracles of the third instar had three separate spiracular slits with incomplete and highly pigmented peritreme (Fig. 6). Similarly, Omar (2002) also recorded the characteristic features like anterior spiracles, cephalopharyngeal skeleton, and latero-dorsal spines of third instar specimens of *C. megacephala* while the comparison of the buccal hook of *C. megacephala* and other allied species was documented by Prins (1979).

During metamorphosis of the third instar into pupa, the cuticle of the third post-feeding larvae was tanned forming a hard-oblong cast known as puparium. The puparium of *C. megacephala* was typically of coarctate type. Colour of the puparium changed from creamy white (early puparium) to light yellow brown and then finally to brown. Clearing technique diluted the outer shell of puparia specimen enabling observation of two anterior spiracles at top ends of trapezoid-shaped anterior plate (Fig. 7) and posterior spiracles having three extremely pigmented dark brown spiracular slits and a highly pigmented button surrounded by peritreme (Fig. 8), which are significant characters for identification. During the investigations, the puparia can be found in or around cadaver, or on the clothing of the deceased (Turchetto et al., 2001 ; Sukontason et al., 2006). The pupal stage being the lengthiest in duration can meet the requirements in PMI estimation (Zehner et al., 2006). The depiction of the posterior spiracles of *C. megacephala* puparia are in consonance with the

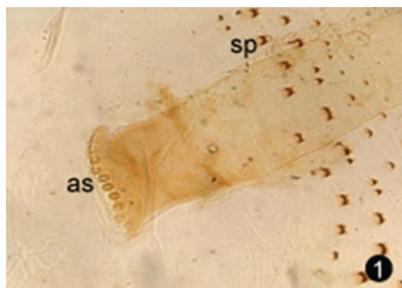


Fig. 1. Anterior spiracles sticking out from second thoracic segment and spines  
 Legend: as: anterior spiracle; sp: spines

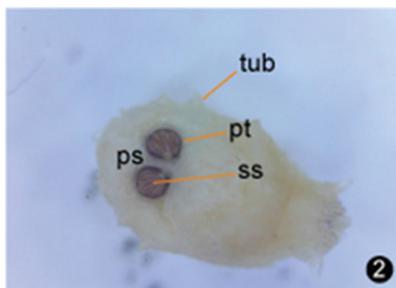


Fig. 2. Posterior end of larva Legend: ps: posterior spiracle; ss: spiracular slit; tub: tubercles; pt: peritreme

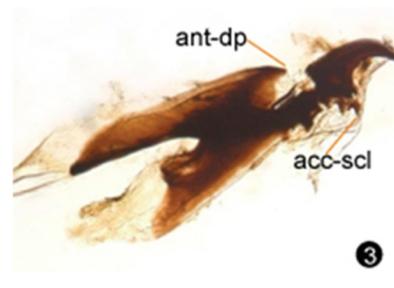


Fig. 3. CPS of second instar larva  
 Legend: acc-scl: accessory sclerite; ant-dp: anterior dorsal process

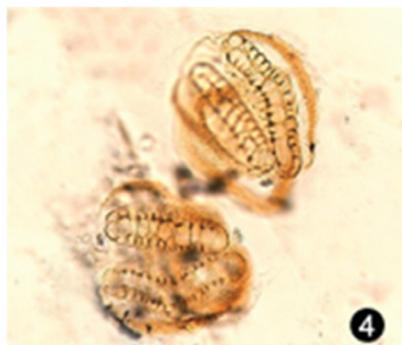


Fig. 4. Posterior spiracle of second instar

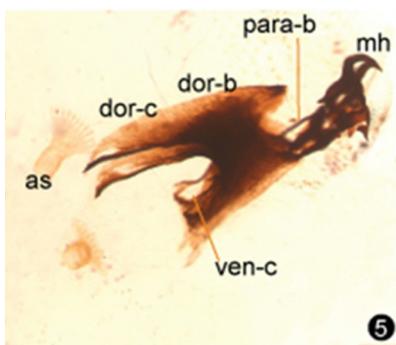


Fig. 5. CPS of third instar larva Legend: as: anterior spiracle; dor-c: dorsal cornu; dor-b: dorsal bridge; para-b: parastomal bar; mh: mouth hook; ven-c: ventral cornu



Fig. 6. Posterior spiracle of third instar

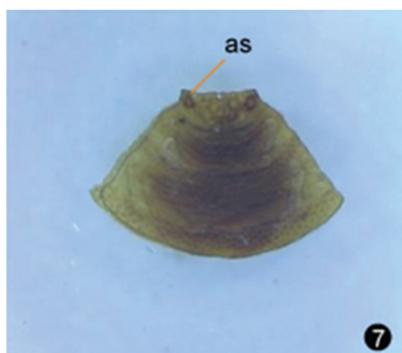


Fig. 7. Anterior part of puparium of *C. megacephala* (treated with 20% KOH for two days). Legend: as: anterior spiracle

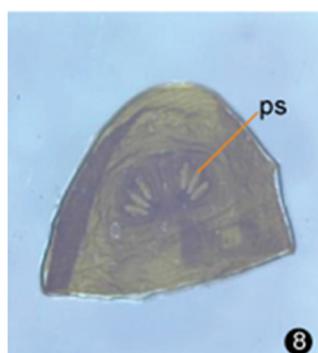


Fig. 8. Posterior part of puparium of *C. megacephala* (treated with 20% KOH for two days). Legend: ps: posterior spiracle

studies performed by Ishijima (1967) and Sukontason et al. (2007). However, puparia are strikingly similar in general appearance and identification of the species, hence using puparium requires much skill and experience (Giordani et al., 2018).

Since, morphological identification can be challenging and at times unattainable due to resemblances among species, particularly in immature stages and sometimes

in adults too (Harvey et al., 2003; Cooke et al., 2018), molecular identification of the fly was also carried out in the present study. PCR amplification of COI of *C. megacephala* was amplified using specific primers which resulted in amplicon of 700 bp. The amplicon was cloned in pTZ57RT vector and the positive clones were confirmed by PCR with specific and universal M13 primers which resulted in ~700 and ~850 bp amplicons respectively. The nucleotide sequence blast

analysis identified the fly as *C. megacephala* (Accession No. MT679549). It displayed 99.39 % identity with sequence of same species collected from Maharashtra, India (Accession No. MG816777.1) (Table 1). *C. megacephala* and other diptera have also been identified using barcoding in China (Qiu et al., 2017), Taiwan (Chen et al., 2004; Sontigun et al., 2018), Egypt (Salem et al., 2015), Saudi Arabia (Mashaly et al., 2017), Caribbean (Yusseff-Vanegas and Agnarsson, 2017), northern and southern part of India (Bharti and Singh, 2017) and Tamil Nadu, India (Ramraj et al., 2014).

The phylogenetic tree was prepared for *C. megacephala* nucleotide sequences from different regions of India and one samples each from China, Korea, Malaysia and Thailand. All the sequences were aligned and 538 bp were used to develop the phylogenetic tree. The phylogenetic tree grouped Jaipur population separately than other samples (Fig. 9). All other samples except two from Kerala were grouped together. Although Jaipur population was different from other populations, but the maximum genetic distance was 0.006. Therefore, it is evident that genetic variation exists in Jaipur population, but this species is not diverse from *C. megacephala*. However, more populations are

required for study to catalogue the genetic diversity of *C. megacephala* in future. Similarly, Kavitha et al. (2012) identified maggot specimens of *C. megacephala*, *C. rufifacies* and *C. nigripes* recovered from human cadavers using morphological identification and confirmed their presence through phylogenetic analyses.

The present study extends a step towards morphological characteristics and DNA barcode archive of *C. megacephala* from Jaipur, Rajasthan which could serve as a supportive input to forensic entomology investigations in this region. Therefore, it is strongly recommended to integrate DNA barcoding reference database of forensically important blowflies from diverse geographical regions of India to make forensic entomology further valuable.

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Table 1. Sequence homology of mt COI region of *C. megacephala*

Seq.	Description	Max. score	Query coverage	Identity	GenBank Accession No.	Country
1	<i>Chrysomya megacephala</i>	1194	100%	99.39%	MG816777.1	Maharashtra, India
2	<i>Chrysomya megacephala</i> strain DI212 isolate Binzhou 2	1194	100%	99.39%	MK075818.1	China
3	<i>Chrysomya megacephala</i> strain DI212 isolate Dezhou 2	1194	100%	99.39%	MK075815.1	China
4	<i>Chrysomya megacephala</i> strain DI212 isolate Heze 1	1194	100%	99.39%	MK075813.1	China
5	<i>Chrysomya megacephala</i> strain DI212 isolate Heze 3	1194	100%	99.39%	MK075811.1	China
6	<i>Chrysomya megacephala</i> strain DI212 isolate Liaocheng 1	1194	100%	99.39%	MK075809.1	China
7	<i>Chrysomya megacephala</i> strain DI212 isolate Qingdao 3	1194	100%	99.39%	MK075804.1	China
8	<i>Chrysomya megacephala</i> strain DI212 isolate Yantai 1	1194	100%	99.39%	MK075802.1	China
9	<i>Chrysomya megacephala</i> strain DI212 isolate Yantai 2	1194	100%	99.39%	MK075801.1	China
10	<i>Chrysomya megacephala</i> strain DI212 isolate Zaozhuang 2	1194	100%	99.39%	MK075798.1	China

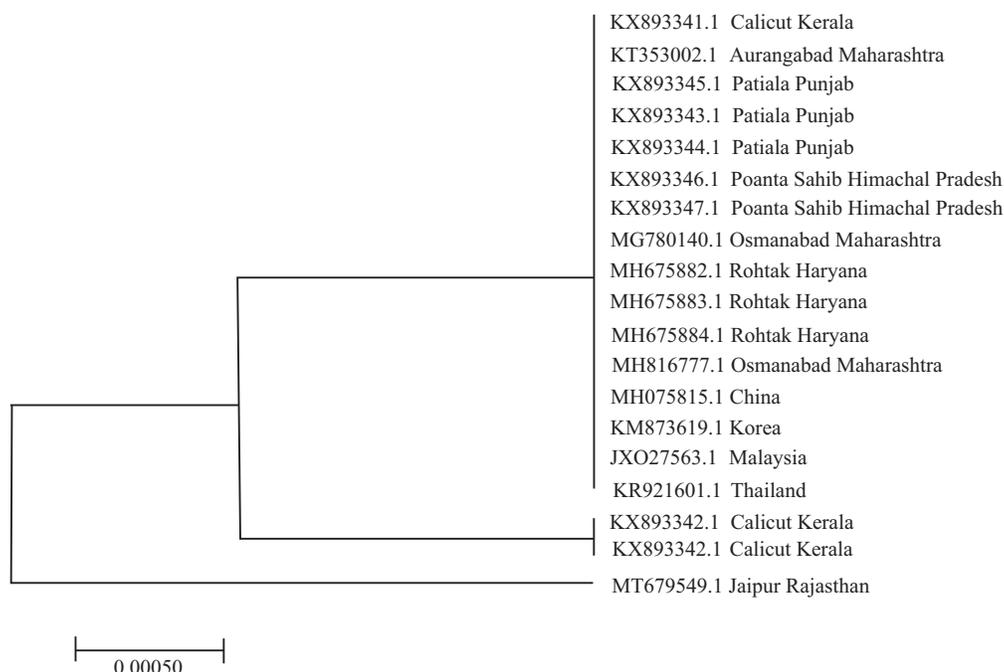


Fig. 9. Molecular phylogenetic analysis of *C. megacephala* by maximum likelihood method (total of 538 positions in the final dataset). Evolutionary analyses were conducted in MEGA7)

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