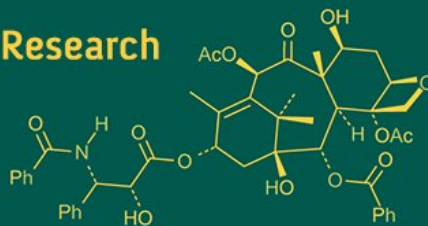


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Forensically important blowfly species (diptera-calliphoridae) recovered from carcasses of wild animals of peninsular region

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Abstract

Dipteran flies play important role in postmortem intervals of badly putrefied carcasses. Successional waves and eggs laying habit on the corpse orifices are used for forensic investigations. The blowfly's succession on the corpse helps for collecting evidence to legalize the poaching stances. The maggot collected from 44 wild animal carcasses including carcasses of carnivores and herbivores including non-human primates (2020-2025) including protected and non-protected forest areas for entomological inputs pertaining to post-mortem intervals evaluation. The maggots were processed for morphological studies and molecular identification including retrieval of host DNA from the gut contents of maggots. Identification of instars stages and respiratory spiracles of maggots were conducted using standard protocols for identification of species of blowflies through DNA extraction using DNeasy blood and tissue kit and amplified through mitochondrial cytochrome oxidase Subunit-I (COI) gene. The PCR products were sequenced unidirectional and confirmed with basic local alignment search tool (BLAST). In different wild animal carcasses utmost 5 species of blowflies were reported. The morphology and molecular identification of maggots revealed highest involvement of *Chrysomya rufifacies* (38.6%), followed by *Chrysomya megacephala* (29.5 %), *Chrysomya nigripes* (15.9 %), *Hemipyrella ligurriens* (6.8%) and lowest of *Lucilia illustris* (4.5%). The study also emphasized that cool chain maintenance may ease the retrieval of host DNA from the gut contents of maggots for forensic uses.

Keywords: Blowfly, maggots, *chrysomya*, instars, forensic entomology, wildlife

Introduction

Forensic entomology substantiates the post-mortem examinations explicitly for collection of evidences to legalize the matter before courts for justice as poaching became threat to global biodiversity and ecosystem stability. Scientific evidence collections confined to prosecution and conviction or defending the victims needs systematic procedure to unfold the facts (Chavan *et al.* 2023) ^[3]. Traditional forensic techniques have their own limitations in addressing the challenges hence poaching stances often overlooked owing to insufficient evidences and lack of scientific investigations (Rana and Kumar, 2023) ^[14]. Forensic entomology offers a powerful tool for estimating the post-mortem interval or time since death, as well as providing insights into the circumstances surrounding a wildlife crime. Subsequently, analyzing the insect communities associated with animal carcasses or remains their developmental phases on the corpse could provide valuable information to investigators, aiding advantage to reconstruct the event and plausible explanations behind the crime scene. The application of forensic entomology extends beyond merely estimating the time elapsed since death that can also reveal crucial details about involvement of specific toxins or poisons in the animal's demise (Bansode *et al.* 2025) ^[2]. This multidisciplinary approach leverages insect biology, ecology, and behavior to provide a comprehensive understanding of the events leading up to death of an animal, thereby strengthening the evidence base for wildlife crime investigations.

Blow fly maggots (Diptera: Calliphoridae) are one of the prominent biological tools plays important role for estimation of post mortem intervals, post mortem transfers or presence of drugs or poisons on the carcass (Wang *et al.* 2021) ^[19]. Blow flies of Calliphoridae family also assist in decomposition process and are the earliest insects to infest a corpse

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(Martin-Vega *et al* 2017). Various arthropods, primarily Diptera (flies) and Coleopteran beetles feed on corpse and complete their larval development and their successional waves could be used for interpretations leading to elapse time since death (Simon 2019) [17]. The insect found on the cadaver provides an important source of information not only for elapse time of death but also inform about saprophytic microorganisms responsible for rapid decomposition of carcass (Sharma *et al.* 2015) [15]. Blow fly maggots (Diptera: Calliphoridae) plays important role for estimation of post mortem intervals (Rajgopala *et al.* 2014). It also assists 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 (Radhakrishnan *et al.* 2012) [11]. The use of forensic entomology in the field of wildlife crime is still insufficient. Accurate identification of maggot species is usually the first priority in a forensic analysis of the entomological evidence. The most important stances for estimation of elapse time since death fixed on priorities like insect species, growth rate, arrival time, and position within the order of succession. However, other relevant physiological or natural history factors can only be attributed for proper identification (Simon, 2019) [17]. Morphological identification of blow flies is rather complicated owing to phenotypic similarities amongst sub-species (Rajgopala, *et al* 2012) [13]. Hence, bar-coding of forensically important blowflies is reliable and rapid for species involvement of Dipteran flies.

The potentials of mitochondrial cytochrome oxidase subunit (COI) is well established gene for species identification but some time it is not effective hence ribosomal internal transcribed spacer (ITS 1 and ITS2) being used for species identification (Oliveira *et al.* 2011) [11]. However, in the present study Mitochondrial COI gene is used for species identification of recovered maggots.

Materials and Methods

The present investigation had done in different tiger reserves of Madhya Pradesh and veterinary physicians along with forest officers contributed for collection of biological samples pertaining to post mortem examinations as per the scheduled plan and commitment. The standard protocols were followed for collection of biological samples to analyze the facts behind the carcass spotted.

Collection of maggots: The maggot collected from 44 wild animal carcasses including carcasses of carnivores and herbivores including non-human primates (2020-2025

including protected and non-protected forest areas with the approval of PCCF wildlife (Government of M.P.). The maggots were collected from each carcass subjected to morphological examinations, identification of developmental phase of the maggot and kept for molecular identification using standard protocols (Rajgopala, *et al.* 2012) [13].

DNA extraction: Maggots tegument DNA was extracted using DNeasy Blood and 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 extraction and a fragment of 229bp of COI gene was amplified. However, mitochondrial DNA was amplified on 229 base pair nucleotide of the mitochondrial cytochrome oxidase subunit I. The primers used in the study were designed using the guidelines (Li *et al.* 2011) [7] based on the description: C1-N-2800 (5'-CATTCAAGYCTGTGTAAGCRTC-3') and C1-J-2495 (CAGCTACTTTATGAGCTTTAGG). The PCR mixture contained - 12.5µl of Master mix 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°C followed by 35 cycles of 94°C for 1 minute, 45°C for 1 minute and 72°C for 1.5 minutes. A final extension period of 1 minute at 72°C was used, which was followed by cooling at 4°C. The PCR products were separated electrophoretically on 1% agarose gel and visualized after ethidium bromide staining and DNA sequences were matched through nucleotide BLAST for phylogenetic studies.

Result and discussion

Overall occurrence of blowfly species on carcasses of 44 wild animals were reported as *Chrysomya rufifacies* (38.6%), *Chrysomya megacephala* (29.5 %), *Chrysomya nigripes* (15.9 %), *Hemipyrella liguriens* (6.8%) and lowest of *Lucilia illustris* (4.5%). in different animal carcasses (Table 1 and Fig 1). Species specific susceptibility of blowfly is not seen while fly comes on the corpse without preference as seen common infestation of maggots either in herbivores or carnivores including non-human primates. Similar observation recorded on forensically important blowflies of *Chrysomya megacephala* and *C. rufifacies* (Yang and Shiao, 2012 and Silva *et al.* 2026) [16, 21].

Table 1: Prevalent species of blow flies reported in carcasses of wild animals

SN	Area	Host species	No examined	Blowfly species found
1	Kanha Tiger Reserve	Tiger (<i>Panthera tigris tigris</i>)	4	<i>Chrysomya rufifacies</i>
		Leopard (<i>Panthera pardus</i>)	4	<i>Chrysomya rufifacies</i>
		Barasingha (<i>Rucervus duvaucelii</i>)	2	<i>Chrysomya rufifacies</i>
		Chital (<i>Axis axis</i>)	2	<i>Chrysomya rufifacies</i>
		Sambar (<i>Rusa unicolor</i>)	2	<i>Chrysomya rufifacies</i>
2	Satpura tiger Reserve	Tiger (<i>Panthera tigris tigris</i>)	2	<i>Chrysomya megacephala</i>
		Leopard (<i>Panthera pardus</i>)	2	<i>Chrysomya nigripes</i>
		Sambar (<i>Rusa unicolor</i>)	2	<i>Hemipyrella liguriens</i>
		Langur (<i>Semnopithecus entellus</i>)	2	<i>Chrysomya megacephala</i>
3	Panna Tiger Reserve	Tiger (<i>Panthera tigris tigris</i>)	3	<i>Chrysomya rufifacies</i> , <i>Hemipyrella liguriens</i>
		Leopard (<i>Panthera pardus</i>)	3	<i>Hemipyrella liguriens</i> , <i>Chrysomya nigripes</i> , <i>Chrysomya megacephala</i>
		Sambar (<i>Rusa unicolor</i>)	2	<i>Lucilia illustris</i>
		Chital (<i>Axis axis</i>)	2	<i>Chrysomya megacephala</i>
4	Pench Tiger Reserve	Tiger (<i>Panthera tigris tigris</i>)	2	<i>Chrysomya nigripes</i>
		Leopard (<i>Panthera pardus</i>)	2	<i>Chrysomya nigripes</i>
		Sambar (<i>Rusa unicolor</i>)	2	<i>Chrysomya megacephala</i>
5	Bandhavgarh Tiger Reserve	Tiger (<i>Panthera tigris tigris</i>)	2	<i>Chrysomya rufifacies</i>
		Leopard (<i>Panthera pardus</i>)	2	<i>Chrysomya megacephala</i>
		Chital (<i>Axis axis</i>)	2	<i>Chrysomya megacephala</i>

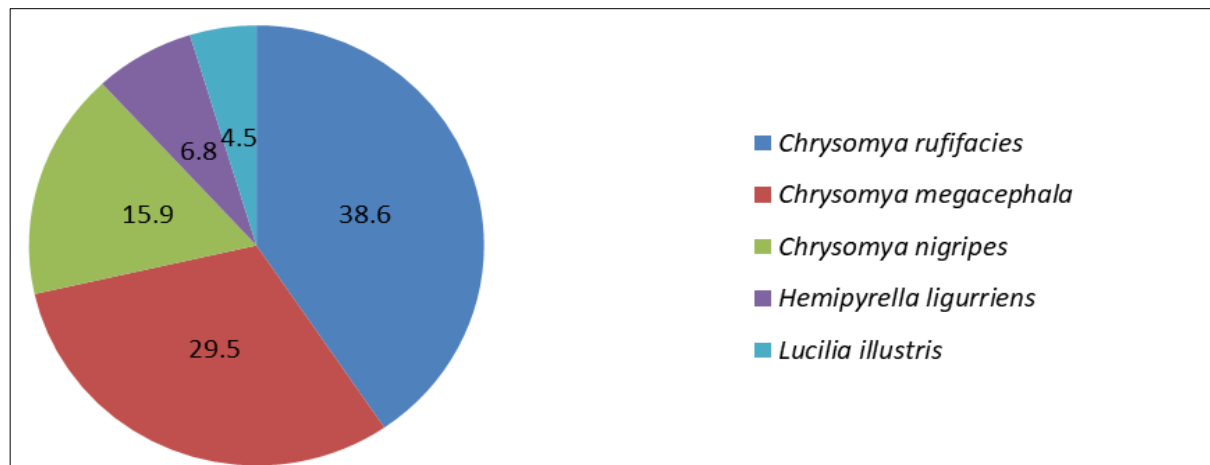


Fig 1: Occurrence of blowfly maggot species recovered from the carcasses of wild animals during postmortem examination

Molecular analysis: The maggots collected from the carcasses of wild animals from different zones of protected forest area were analyzed morphologically and also molecularly. The nucleotide mega blast confirmed the prevalence of 5 blowfly species viz., *Chrysomya rufifacies*, *Chrysomya megacephala*, *Hemipyrella ligurriens*, *Chrysomya nigripes*, *Lucilia illustris*. The phylogenetic trees were constructed based on COI (partial sequence). The

reference sequences were obtained from Genbank for the comparative purpose from previously recorded blowfly sequences namely *Chrysomya rufifacies* (AH015276.2), *Chrysomya rufifacies* (DQ328666.1), *Hemipyrella ligurriens* (JN604563.1), *Hemipyrella ligurriens* (AY842614.1), *Chrysomya nigripes* (JN014863.1), *Chrysomya nigripes* (FJ195379.1), *Lucilia illustris* (KT272845.1) and *Lucilia illustris* (MT584139.1)

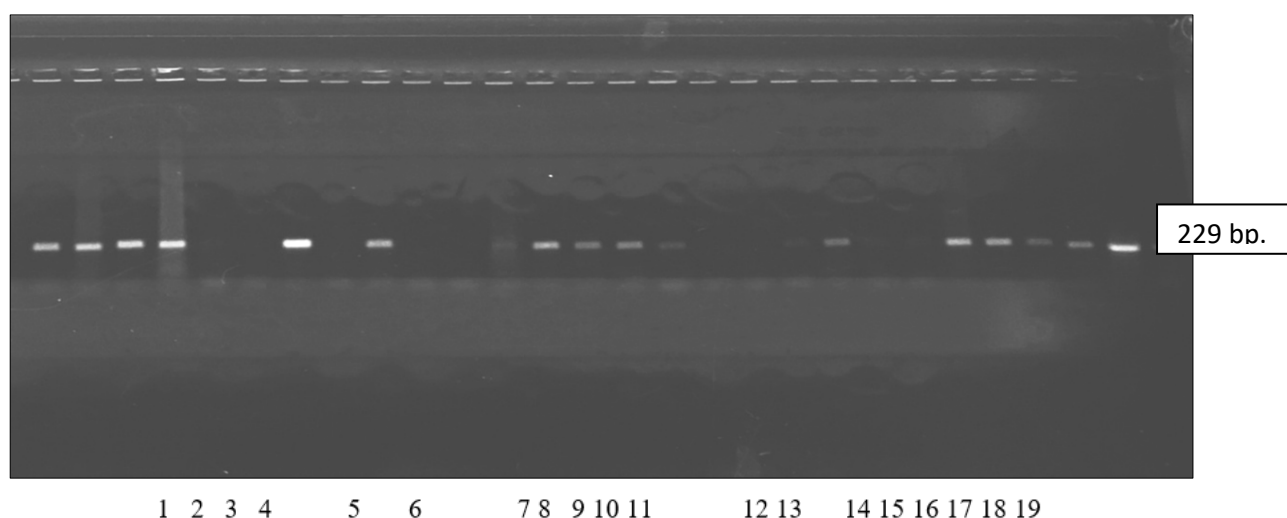


Fig2: Showing electrophoretic separation of the DNA amplified fragment of cytochrome subunit I (COI). Lane 1-19 shows the DNA band at 229 bp of the maggots collected from the carcass of different wild animals

Calculation of elapsed time of death: Calculation of elapsed time of death was done by following the standard protocol and maggot's instars identification through their posterior spiracles and teguments. The maggots were processed and permanent mount of the different stages of maggots were study to compare the maggots collected from carcasses of the wild animals from different tiger reserves of Madhya Pradesh. Overall 44 carcasses were incorporated the study including carnivores and herbivores. The species identification of blowflies using DNA characterization is often in homicidal cases (Chen *et.al.*2004; Kondakci *et al.*2009) [6] and seldom in wild animals (Anderson, 1999) [1, 4] while Watson and Carton (2005) [20] initiated forensic entomological diagnosis in wildlife crime investigation with identification of blowfly species recovered from carcasses Deer Alligator and Swine from Louisiana. Subsequently However, From India, studies have identified *Chrysomya albiceps* in Sambar deer (*Russa unicolor*) on the basis of

morphology (Radhakrishnan *et al.* 2012) [11] while Singh *et al.* (2020) [18] identified the species of blowflies on the carcass of royal Bengal tigers during postmortem examinations. Furthermore, in the present study both morphological identification keys as well as molecular characterization methods had applied for species identification of recovered maggots using COI gene of Mitochondrial DNA. Subsequently, data base has generated for wildlife forensics recovered from 44 carcasses of wild animals covering Tiger, Leopard, Barasingha, Sambar and Langur from different Tiger Reserves of Madhya Pradesh for knowing the prevalent species of blowflies comes earlier on the corpse of wild animals by applying morphological identification tool followed the guidelines of Rajgopala *et al.* (2012) [13] as well as molecular characterization of blowflies maggots from tegument using standard protocols of Linville *et al.* (2002) [8]. The present study is also providing comprehensive account on wildlife forensic

leading to death investigation in protected and non-protected forests area of Madhya Pradesh.

Occurrence of blowfly species: Overall 5 blowfly species i.e. *Chrysomya rufifacies*, *Chrysomya megacephala*, *Hemipyrella ligurriens*, *Chrysomya nigripes*, *Lucilia illustris* of Calliphoridae are prevalent in different Tiger Reserves of Madhya Pradesh. The species *Chrysomya rufifacies*, *Chrysomya megacephala*, *Hemipyrella ligurriens*, are found throughout the year, irrespective of the temperature and relative humidity. Whereas *Chrysomya nigripes* is prominently found in the higher temperature and in contrast to that *Lucilia illustris* was found when the temperature was comparatively on the lower side. Present investigation is identical to the study of Khullar *et al.* (2016) [5] identified 6 species of blowflies in India using short COI marker while Martín-Vega *et al.* (2017) observed early colonization of blowflies in experimental study using pig carcass.

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Conflict and ethical approval: no matters of conflict, as the maggots were collected from carcasses of wild animals including carnivores, herbivores and non-human primates.

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