Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(1): 13-19

Received: 03-11-2016
Accepted: 04-12-2016

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Molecular identification of forensically important blowflies (Diptera: Calliphoridae) with a record of a new species from Maharashtra India

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Abstract
The utility of Cytochrome C Oxidase subunit I gene (COI) DNA barcode for the identification of four species of forensically important blowflies of the genus *Chrysomya* (Diptera: Calliphoridae), from Maharashtra, India, was tested to confirm the morphological identification. And in molecular identification, fragment of 658 bp of COI gene was sequenced of four specimens. In the genus of *Chrysomya*, the means of the nucleotide composition frequency distribution were G= 15.66%, C =15.5%, A= 30.55% and T= 38.3%. In the phylogeny report it was shown that *Chrysomya saffranea* and *Chrysomya megacephala* are close ancestors and monophyletic which means that they are genetically similar but the morphological appearance of these species can be distinguished. *Chrysomya* sp. and *Chrysomya rufifacies* are also monophyletic; both are genetically close with 0.3% distance. And both *Chrysomya* sp. and *Chrysomya* rufifacies are slightly similar morphologically, and both are hairy maggot species, but they differ from each other in some of the morphological marks. So we consider the *Chrysomya* sp. as a new species and named as *Chrysomya indiana*.
The result displays the utility of COI gene as an identification marker, since the nucleotide variations give a clear and powerful difference between species. Four new COI gene sequences have been added to GenBank and out of them one was consider as a new species, therefore, these sequences can be of concern for correct species identification for future workers.

Keywords: Calliphoridae identification**,** molecular identification, COI, new species *Chrysomya indiana*

Introduction
To estimate the post-mortem interval in Forensic Entomology medico-criminal investigations, accurate identification of insects collected from a corpse is required. However, most entomological evidence are strongly reliant on accurate species identification, because identification allows the proper developmental data and distribution ranges to be applied in criminal investigations [1, 2]. Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for phylogenetic studies in animals because of its sample genomic structure [1, 3-6].
Study of mitochondrial DNA (mtDNA); particularly, Cytochrome C Oxidase subunit I gene (COI) appeared to be a useful tool in species identification among the subfamilies of Calliphoridae and Sarcophagidae [1, 7-13]. The mtDNA offers several advantages over nuclear DNA: the latter undergoes relatively slow mutation rates compared to mtDNA. Hebert *et al*., [14] said that COI has two important advantages: (1) COI appears to have a greater range of phylogenetic signal than any other mitochondrial gene; the evolution of this gene is quick enough to tolerate the discrimination of not only closely allied species, but also phylogeographic groups within a single species, and (2) the universal primers for this gene are very vigorous enabling the recovery of its 5' end from among the representatives of most, if not all, animal phyla. So identification would require a much longer nucleotide sequence than is necessary with mtDNA. This makes mtDNA a better tool to determine differences in the sequences of closely related species, therefore, useful for molecular identification [15].

Materials and Methods
a) Morphological identification
Collected Calliphoridae species were dissected and identified using morphological characters and identification keys [16-30]. Also some morphological keys of adult fly were studied under the microscope as stated below to follow the identification keys to the different species of

Chrysomya and supported with photos for some parts used in the identification.

In this study we have recorded a new species of Calliphoridae named as *C. indiana*. The most identification characteristics of *C. rufifacies* and *C. indiana* are presented in Figure 1 and 2, respectively.

***Chrysomya indiana* sp. n.**

Type material

HOLOTYPE (male): India: M. S. / Marathwada region/ Aurangabad, (19.9047N, 75.3102E) 02-VIII-2012, PARATYPES: 1 (male) same locality, same data as Holotype. The holotype deposited to the Forensic Entomology Laboratory, Zoology department, Dr. BAMU, Aurangabad

Distribution: Aurangabad city, Maharashtra, India**.**

Etymology: Named after the respect country, India

Identification keys for Chrysomya species

1) Flies not more than 8mm in length; eyes widely separated in male; hypopygium inconspicuous …… *Chrysomya nigripes*

-Flies 10mm in length or longer ……..……..……2

2) Anterior border of wings deeply infuscated ……. *Chrysomya marginalis*

-Wings are entirely hyaline ……..……..3

3) Prothoracic spiracle white or yellow …… 4

-Prothoracic spiracle brown ……..……..... 5

4) Prothoracic spiracle yellow; ventral side of the body is golden color (golden bluebottle blowfly) ……...…. *Chrysomya incisuralis*

- Prothoracic spiracle white…….. 11

5) Femora swollen in both sexes, but more noticeably in male; distance between the eyes in both sexes equal to one-fifth of total width of head, facets small and uniform, frons parallel-sided, parafrontalia black towards vertex, with silver tomentum anteriorly; ocellar bristles very weak dark reddish-black, fronto-orbital bristles absent in female, Legs: dark brown, femora and tibia greatly swollen in male, less so in female, the former metallic in certain lights, segments of tarsi in male clearly demarcated from one another ……..……..……..... *Chrysomya villeneuvi*

-Femora normal 6

6) Eyes in the male more or less closely approximated; male hypopygium more or less inconspicuous.....7

7) Eyes in male separated by four-fifth of width of one eye; male hypopygium large.....…… *Chrysomya phaonis*

8) Parafacialia never brilliant orange 10

- Parafacialia and jowls brilliant orange, the latter clothed with golden hairs 9

9) Face covered with grey tomentum ……..……..…….. *Chrysomya defixa*

-Face dark reddish, jowls grey, black-haired …….....…. *Chrysomya pinguis*

10) No black setulae on facial or parafacial around the vibrissa or, rarely, 2 or 3 present; frontal stripe of female not broader at middle of frons, parallel-sided; facets of eye of male larger above than below, but without any distinct line of demarcation; squamae brown. Larvae have complete bands of spines broad, 'on II-VIII;' band' on IX' slightly narrower and sparser; band on X very narrow and sparse, visible only on cleared specimen; most spines single-tipped but a few in the ventral spine bands double-tipped anterior spiracles with 11-14 processes.……..……..…..... *Chrysomya saffranea*

-At least several, usually many black setulae around vibrissa, on face and parafacial; facets of eye much enlarged above and sharply demarcated from the area of smaller facets below; frontal stripe of female broader at middle of frons, not parallel-sided, Squamae brown ……...……..…… *Chrysomya megacephala*

11) Prothoracic spiracle white or creamy, Adult small (5-6mm long); face and cheeks wholly yellow; male front femur with prominent, long white hairs..... *Chrysomya varipes*

- Prothoracic spiracle white, Adult large (7 to 8 mm long); face and cheeks with dense silvery hairs on dark brown to black surface ……..……12

12a) Anterior spiracle is open, presence of proepisternal seta (stigmatic bristle) (Figure: 1b). Larvae have complete bands of spines on II-IV; narrower band on V; many spines with two or three tips; eight fleshy protrusions per segment on IV-XI, those on V-XI having dark spines on their tips and the third to the sixth protrusions on V-XI being covered with dark, round-tipped scales and the space between the fourth and fifth projections being covered with similar scales; numerous spines only on the tip of the tubercle(Figure: 1c),peritreme of the posterior spiracles are with small gap (Figure: 1f),anterior spiracle with 9-10 processes (Figure: 1e) ……..…… *Chrysomya rufifacies*

12b) Anterior spiracle is closed; absence of proepisternal seta…….. *Chrysomya albiceps*

12c) Anterior spiracle is closed, presence of proepisternal seta (stigmatic bristle) Figure: 2b, numerous spines surrounding the tubercle from the base to the top (Figure: 2c), peritreme of the posterior spiracles are with very small gap or closed (Figure: 1f) …… (New Species) …… *Chrysomya indiana*

b) Molecular identification

Specimens

For barcoding DNA, single specimens of each of the four collected species of Calliphoridae were prepared; *Chrysomya megacephala, Chrysomya saffranea, Chrysomya indiana* (new species), *Chrysomya rufifacies* from different localities (Table 1). Legs were removed from male adult specimens and kept in Eppendorf tube containing 3 ml of absolute alcohol. All instruments used to remove the legs were sterilized with 70% Ethanol, all steps were done in sterile condition.

Extraction of DNA

DNA from each specimen was extracted separately by using QIAamp-DNA Mini kit with the tissue protocol, following the manufacturer's instruction. Tissue samples were cut into small pieces, and placed in a 1.5ml microcentrifuge tube, and 180µl of Buffer ATL.QIAGEN proteinase K was added and incubated at 56 °C overnight until the tissue completely lyses.

Amplification and purification

Gene COI fragment was amplified using Universal primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' 25 base pair and HCO2198 5'-TAAACTTCAGGCTGACCAAAAAATCA-3' 26 base pair. 1µL of template DNA was added in 20µL of PCR reaction solution. The PCR reaction was performed with Initial denaturation at 94°C for 5 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec with a final Extension at 72°C for 10 min. A positive control (*E.coli* genomic DNA) and a negative control were included in the PCR. Unincorporated PCR primers and dNTPs were removed from the PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the LCO1490/HCO2198 primers.

Sequencing reactions were performed using an ABI PRISM Big Dye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

DNA Sequencing

Sequencing forward and reverse was performed on each template using universal primers LCO1490 and HCO2198. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. Sequencing was performed by using ABI 3730xl sequencer (Applied Biosystems).

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of the sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [31]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) [32]. Finally, the program PhyML 3.0 a LRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as the other existing phylogeny programs using simulated data, while being one of order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [33].

The alignment of Diptera sequences was carried out using the ClustalX v2.0.12, and the alignment file for analysis was prepared with BioEdit Sequence Alignment Editor V7.0.5.3. To avoid interferences in the analyses due to the presence of stop codon, the sequences ends were cut. Analysis was, therefore, made with 658 bp from COI barcode fragment, each sequence was submitted to Barcoding of Life Database (BOLD; available online at www.boldsystems.org). All sequences were entered into BOLD under the project name DNA barcoding of forensically important Calliphoridae and Sarcophagidae flies (DBCS).All sequences of the samples were storage and preliminary barcoding analyses were done. Sequences were submitted to NCBI and the GenBank accession Number of the samples was obtained (Table: 1).

Results and Discussion

The COI region proved straightforward to amplify and sequence. A 658-bp fragment of the COI gene from all 7 specimens was successfully sequenced. The alignment of all sequences used in this study showed lack of stop codons which indicated that coamplification of nuclear pseudogenes did not occur and did not show any insertion or deletion. Nucleotide composition frequency distribution within *Chrysomya* shown in Table: 2 which is similar to the previous studies with very small changes. Nelson *et al.,* [34] reported that the mean values of nucleotide composition frequency distribution within *Chrysomya* were A= 30.85%, C= 15.5%, T= 38.3% and G= 15%, While Bajpai *et al*., [35] reported A=31.4%, C=15.4%, T=39.6%, G=14%; these small changes may be because of the variety of species.

According to K2Pmodel the sequence divergence at the COI barcode region among these Calliphoridae in the species level showed the minimum, maximum and the mean sequence distance of 0.0%. While at the genus level they showed minimum distance of 0.0% and the maximum distance of 6.54%, while the mean divergence of 4.3% and the standard error of distance0.49%. While the Pairwise Distance showed the minimum distance of 0.0% and the maximum distance of 6.23%, while the mean distance of 4.1% and the standard error of distance 0.47% (Table: 3).

The distribution of sequence divergence at each taxonomic level according to K2P model the distance between the *Chrysomya saffranea* vs *Chrysomya megacephala* was 0.0% while the distance between *Chrysomya saffranea Chrysomya rufifacies* and *Chrysomya saffranea* vs *Chrysomya* sp. (*Chrysomya indiana*) was 6.203% and 6.541% respectively. While the distance between the *Chrysomya megacephala* vs *Chrysomya* sp. (*Chrysomya indiana*) and *Chrysomya rufifacies* vs *Chrysomya* sp. (*Chrysomya indiana*) were 6.541% and 0.305% respectively. While in the Pairwise Distance model, the distance between *Chrysomya saffranea* vs *Chrysomya megacephala* was 0.0% while *Chrysomya saffranea* vs *Chrysomya rufifacies* and *Chrysomya saffranea* vs *Chrysomya* sp. (*Chrysomya indiana*) was 5.927% and 6.231% respectively. While the distance between the *Chrysomya megacephala* vs *Chrysomya* sp. and *Chrysomya rufifacies* vs *Chrysomya* sp. was 6.231% and 0.304% respectively (Table: 4).

The Barcode Gap Analysis for the distribution of distances within each species and the distance to the nearest neighbor of 4 Calliphorid species has shown the minimum, maximum, mean and standard error divergence of 0%, 0.3%, 0.15% and 0.04% respectively. The Barcode Gap Analysis data for the species comparisons has shown that the *Chrysomya saffranea* is the nearest species to *Chrysomya megacephala* with 0.0% distance, and the *Chrysomya* sp. (*Chrysomya indiana*) is the nearest species to *Chrysomya rufifacies* with 0.3% distance.

Phylogenetic tree has shown that *C. saffranea* is very closely related species with *C. megacephala*; they share most common ancestor characters. Similarly *Chrysomya* sp. (*Chrysomya indiana*) with *Chrysomya rufifacies* are common ancestors with distance less than 0.5% (Figure: 3).

In this study we have reported the molecular identification of four Calliphorid species using barcoding of mtDNA technique, and Cytochrome COxidase subunit I (COI) gene was used to confirm the morphological identification of four of Calliphorid species. *C. megacephala* and *C. saffranea* are generally regarded as morphologically and genetically similar but ecologically distinct [36-38]. Fahd *et al.,* [39] reported that *C. saffranea* was morphologically similar to *C. megacephala* with some minor identification keys differ from each other, similar identification was reported in Australia [20], but in molecular identification using COI gene no interspecific sequence was observed. *C. saffranea* showed that it is monophyletic with its close sister *C. megacephala*. Harvey *et al*., [8] using COI analyses reported that the maximum interspecific sequence variation between *C. saffranea* and *C. megacephala* was only 0.33% and reported that *C. megacephala* is monophyletic with *C. saffranea* as its sister species. While Wallman *et al*., [40] compared one individual of *C. saffranea* (Queensland) and *C. megacephala* (New South Wales)and reported only 0.403% variation between them, and 0.48% molecular divergences between these two species was reported by Nelson *et al*.,[34,41].

Harvey *et al*., [8] reported that interspecific variation between *C. megacephala* and *C. saffranea* differs by only 0.33% by using COI gene analysis and reported the maximum intraspecific variation of *C. saffranea* and *C. megacephala* was 0.18% and 0.34% respectively percentage of the total of 1167 base pairs of COI.

Wallman *et al*.,[40] also compared one individual of both of *C. saffranea* (Queensland) and *C. megacephala* (New South Wales) and reported only 0.4% variation between them across a variety of barcode regions, including 822 bp of the COI gene and shown that *C. megacephala* monophyletic with

respect to *C. saffranea*.

Also *Chrysomya* sp. and *C. rufifacies* are common ancestor species with maximum interspecific sequence variation0.3%. And the *C. rufifacies* is monophyletic with the respect to *Chrysomya* sp. and morphologically they differ from each other in many of morphological identification keys. So we consider it as a new species and named as *Chrysomya indiana*. This case may be similar to *C. saffranea* and *C. megacephala.*

Table 1: Specimens with collection location and the GenBank accession number

species	GenBank Accession No.	Sample ID	Collection locality Lat/Long
Chrysomya megacephala	KT353002	FZ 10	19.9041, 75.3115
Chrysomya rufifacies	KT353003	FZ 20	19.4824, 75.3785
Chrysomya saffranea	KT353004	FZ 30	19.9032, 75.312
Chrysomya sp. New species (*indiana*)	KT353005	FZ 40	19.9047, 75.3102

Table 2: Nucleotide composition frequency distribution within genus *Chrysomya*

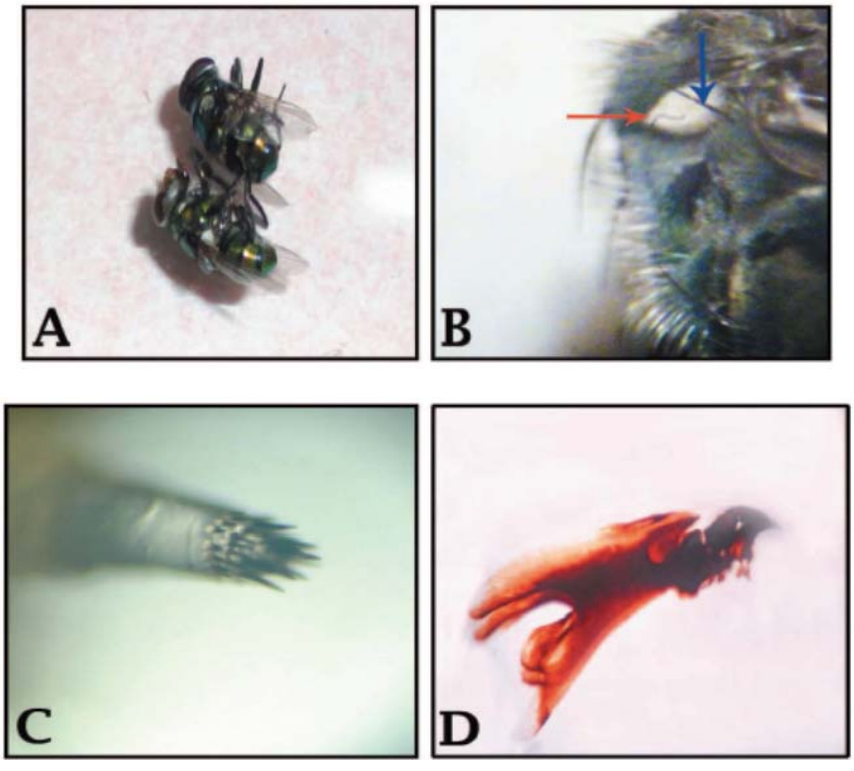
	Main	Max	Mean	SE
G%	15.35	15.81	15.66	0.11
C%	15.35	15.65	15.5	0.09
A%	30.4	30.85	30.55	0.11
T%	38.15	38.45	38.3	0.09

Table 3: The sequence divergence between barcode sequences at the species, genus and family level according to Pairwise Distance and K2P models

Sequence Divergence	n	Taxa	Comparisons	Min Dist. %	Max Dist. %		Mean Dist. %		SE Dist. %	
					Pairwise Dist.	K2P	Pairwise Dist.	K2P	Pairwise Dist.	K2P
Within Species	0	0	0	0	0	0	0	0	0	0
Within Genus	4	1	6	0	5.23	6.54	4.1	4.3	0.49	0.47
Within Family	0	0	0	0	0	0	0	0	0	0

Table 4: The distribution of sequence divergence at each taxonomic level according to Pairwise Distance and K2P models

	Main Dist. %	Max. Dist. % (within the genus level)		Max. Dist. % (within the species level)
		Pairwise Dist	K2P	
Chrysomya rufifacies vs *Chrysomya megacephala*	0	5.927	6.203	0
Chrysomya saffranea vs *Chrysomya megacephala*	0	0.000	0.000	0
Chrysomya saffranea vs *Chrysomya rufifacies*	0	5.927	6.203	0
Chrysomya sp. vs *Chrysomya megacephala*	0	6.231	6.541	0
Chrysomya sp. Vs *Chrysomya rufifacies*	0	0.304	0.305	0
Chrysomya sp. Vs *Chrysomya saffranea*	0	6.231	6.541	0



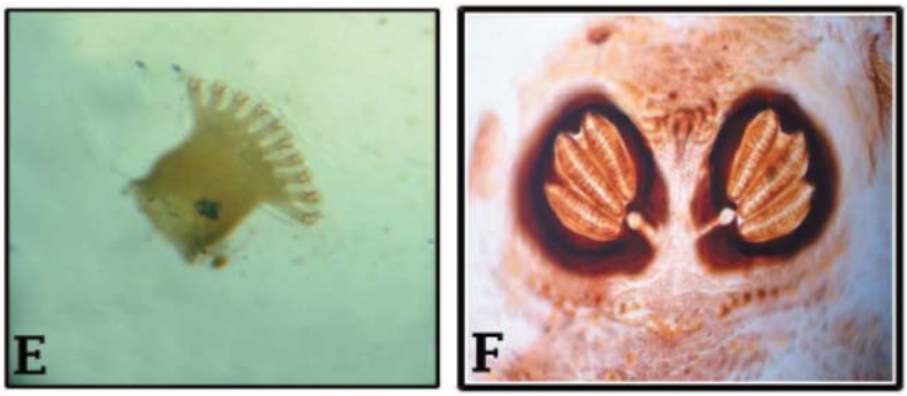


Fig 1: *Chrysomya rufifacies* A- Copulating of pair of *Chrysomya rufifacies*. B- Anterior thoracic spiracle of adult fly of *Chrysomya rufifacies* showing closed spiracle, presence of stigmatic bristle. C- Tubercles of 3rd instar larva. D- Cephalopharyngeal skeleton of 3rd instar larva. E- Anterior spiracle of 3rd instar larva. F- Posterior Spiracle of 3rd instar larva

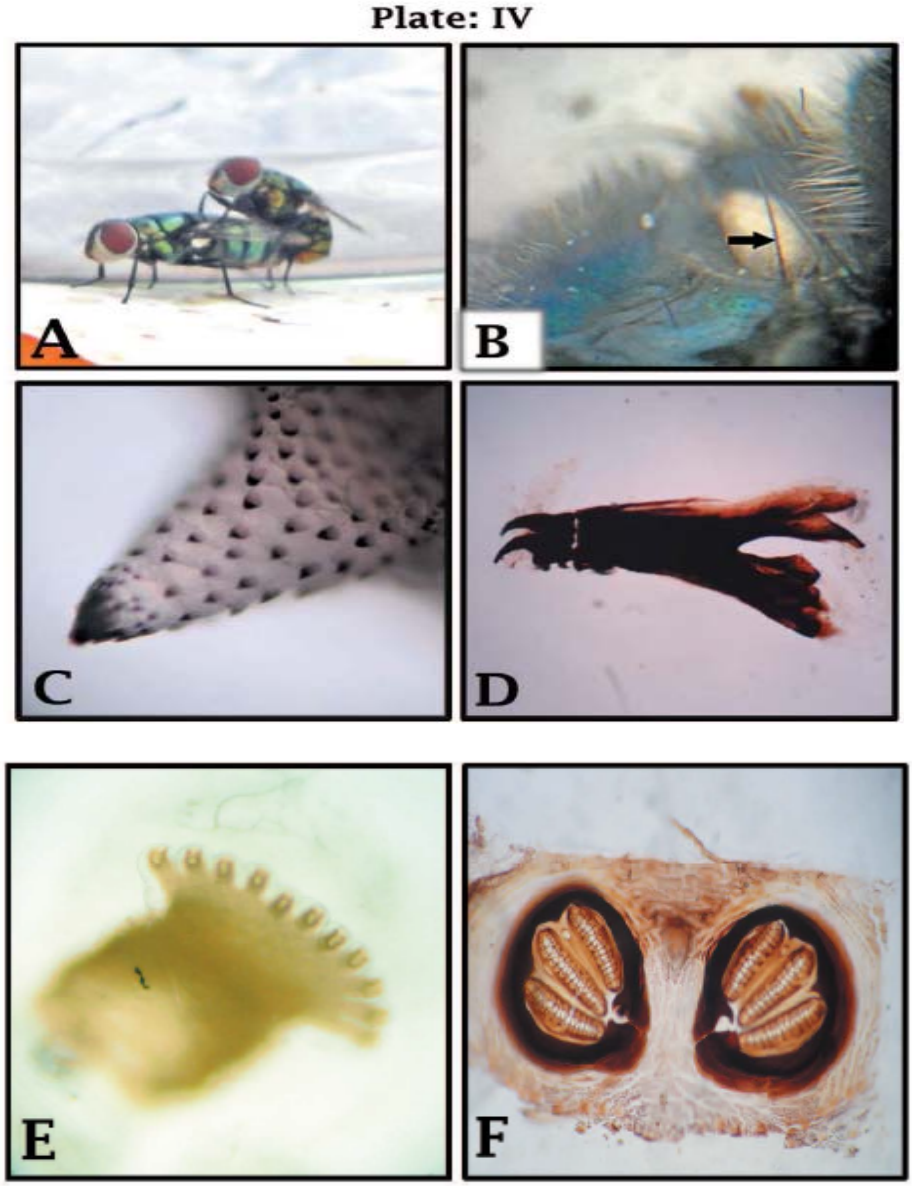


Fig 2: *Chrysomya indiana* (*Chrysomya* sp.) B- Anterior thoracic spiracle of adult fly of *Chrysomya indiana* showing closed spiracle, presence of stigmatic bristle. C- Tubercles of 3rd instar larva. D- Cephalopharyngeal skeleton of 3rd instar larva. E- Anterior spiracle of 3rd instar larva. F- Posterior Spiracle of 3rd instar larva.

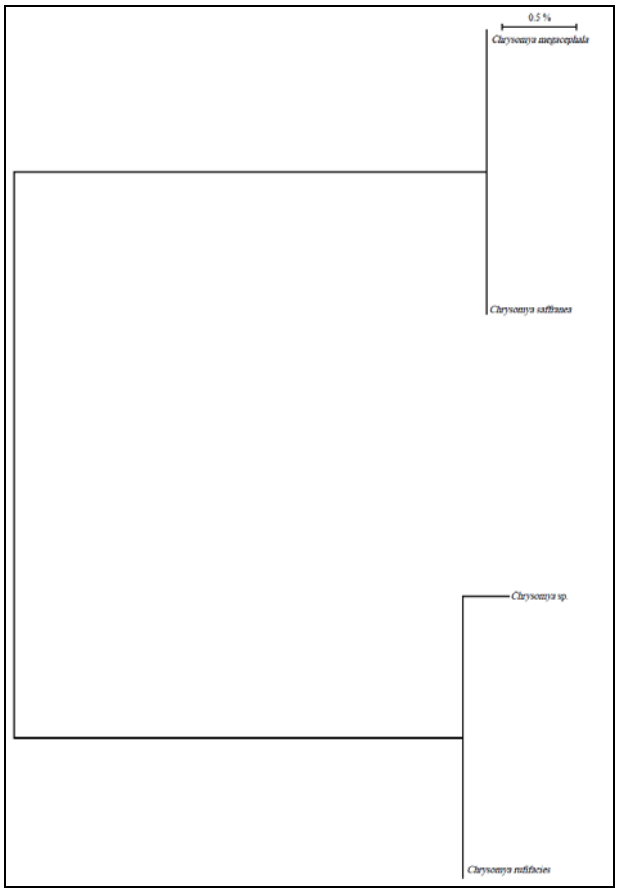


Fig 3: Phylogenetic tree of the samples with the distance between the close ancestor species.

Conclusion

Four species of genus *Chrysomya* were collected, morphological identification have been done using identification keys and molecular DNA barcoding using COI gene. *C. saffranea* and *C. megacephala* are genetically similar and morphologically distinct. Similarly *Chrysomya* sp. and *C. rufifacies* are genetically similar with 0.3% distance and morphologically different from each other. *Chrysomya* sp. is consider as a new species and named as *Chrysomya indiana*, as a forensic importance flies, these species was recovered from animal remains at different location in Maharashtra State, India

Acknowledgement

The authors would like to thank the higher studies and scientific research, Thamar University- Yemen, for the financial support and they want to express their thankful to the Department of Zoology at Dr. Babasaheb Ambedkar Marathwada University. Aurangabad –India for providing the laboratory facility to carry our work in the forensic entomology laboratory.

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