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MOLECULAR TOXONOMY OF FORENSICALLY IMPORTANT CARRION FLIES USING mt (DNA COI)

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ABSTRACT

Identification of forensically important carrion flies is made challenging by the lack of taxonomists and morphological identification keys. The eggs or larvae are difficult to identify morphologically, and in such cases, molecular technique, DNA Barcoding specific to mt (DNA COI) provides a genuine substitute. The mtCOI of two Calliphoridae species, *Chrysomya megacephala, Chrysomya rufifacies* and one Sarcophagidae species, *Sarcophaga dux* are examined in the present study for molecular identification.

Key words: Molecular identification, *Chrysomya megacephala*, *Chrysomya. rufifacies*, *Sarcophaga dux*, forensic, maggots, carrion flies, mtDNA.

Forensic entomologists use arthropods to determine the postmortem interval (PMI) and the timing of the earliest insect colonisation of humans or other animals. The assessment of PMI can be aided by insect evidence found at crime scenes (Byrd and Tomberlin, 2019). The perinatal stages of insects are often related to species identification, which can be challenging because larval features alone do not provide precise and sufficient identification keys. According to Byrd and Castner (2010), many of these prenatal stages in various animals are yet unknown. Only a few species may be identified using morphological identification keys (Velasquez et al., 2010). As an alternative to morphological identification, mtDNA sequence data can be used to identify immature forms of the species (Hebert et al., 2003). DNA barcoding was suggested by Kress et al. (2005) for accurate identifications of species. One of the first taxonomists to make considerable use of DNA sequences for identifying and defining species was the Dipteran taxonomist. For Dipterans, a variety of molecular markers are used (Wells and Stevens, 2008). A forensic entomologist can benefit from molecular analysis to correctly identify entomological evidence (Bharati and Singh, 2017). The present study focused on the few Calliphoridae and Sarcophagidae flies of Osmanabad, Maharashtra, India.

MATERIALS AND METHODS

A fresh liver sample was purchased from a local slaughterhouse, and when putrefied, it was exposed to air to attract flies. Various animal remains were also used to collect the larvae and adult flies. An insect-catching net was used to catch flies. Flies were collected, brought to the laboratory, raised, and fed diluted honey and raw liver. Fresh liver was used as an oviposition site for females. Until the post-feeding phase, the maggot cultures were fed raw liver. The pupae were placed in 500 ml beakers with 2 cm of dry soil. The adult flies were raised in a rearing box (25x 25x 45 cm) and pure culture was established by separating the eggs or larvae of one female for identification and other experiments. The Magnus MS-224 stereoscopic microscope was used to morphologically analyze these adults and maggots. Several stages of Calliphoridae and Sarcophagidae species were dissected and identified using morphological traits and previously published identification keys. For molecular analyses the samples 1 = *Sarcophaga dux*, 4 = *Chrysomya megacephala*, and 14 = *Chrysomya rufifacies* each were used providing a unique sample ID. DNA barcoding was performed on the thorax of an adult flies. The ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with (FS enzyme) AmpliTaq®DNA polymerase, Montage PCR cleanup kit, QIAamp® DNA small genomic isolation kit, and ABI 3730xl sequencer were used (Applied Bio-Systems). DNA sequencing programs for blasting and alignment (Muscle 3.7, Mega Blast software, Gblocks 0.9b, PhyML 3.0 aLRT, ClustalX v2.012, and Bio Edit Sequence Alignment Editor V7.0.5.3) were used. LCO-1490 GGTCAACAAATCATAAAGATATTGG. 25 base pairs and HCO-2198 T 26 base pairs primers were used. The NCBI blast similarity search tool was used to look up the

18S RNA sequence, and following phylogenetic analysis, closely related sequences, and multiple sequences were performed. The Muscle 3.7 programs were used to align many sequences (Edgar, 2004). Gblocks 0.91b was used to process the aligned sequences that were generated. This G block reduces alignment noise by removing diverging and uneven alignments (Talavera and Castresana, 2007). Finally, PhyML 3.0 aLRT and the HKY85 substitution models were used to conduct phylogenetic analysis and TreeDyn 198.3 was employed to render the trees (Dereeper et al., 2008).

RESULTS AND DISCUSSION

GenBank accession numbers were obtained for each sample of *C. megacephala* (MG816777), *C. rufifacies* (MG816778), and *Sarcophaga dux* (MG816779). The mean and maximum intraspecific values for each species are compared to their closest relatives- *C. megacephala* is closest to *S. dux, C. rufifacies* is closest to *C. megacephala,* and *C. rufifacies* to *C. megacephala.* The closest species distance given by *C. megacephala* and *C. rufifacies* is 7.13, whereas *S. dux* indicates a distance of 11.49. While the maximum intraspecific distance was zero, the mean intraspecific distance did not apply to any of the three forensic flies. Sequence analysis using a species database showed a 100% match to the *C. megacephala* species, which was identified visually as sample ID 4. A=30.4%, T=37.4%, G=16.0%, and C=16.2% make up the nucleotide frequency distribution of *C. megacephala.* Sequence analysis using a species database showed a 100% match to the *C. rufifacies* species, which was identified visually as sample ID 14. The mean nucleotide composition frequency of *C. rufifacies* was A = 30.4%, T = 38.1%, G =15.8%, and C =15.7%. This work led to the discovery of the genus *Sarcophaga*, which is where the recovered sarcophagid sample from a species identified morphologically as *S. dux* and given sample ID number 1 comes from. The top 99 species in a species database used to identify sequences showed a 100% match to *S. dux*. Within the *S. dux* genus, the mean nucleotide frequency distribution is A = 30.8%, T = 37.4%, G = 16.1%, and C = 15.6%.

Phylogenetic analysis of *C. megacephala*, *C. rufifacies,* and *S. dux* shows 100% matches (Figs. 1-3). Sukontason et al. (2003) concluded that *C. rufifacies* and *C. megacephala* have strong similarities despite using scanning electron microscopy, and DNA barcoding is a valuable tool. Many forensically significant dipteran species' eggs or larvae are extremely difficult to differentiate morphologically (Benecke and Seifert, 1999, Liu and Greenberg, 1989). This is owing to the fact that adult arrival timings, egg length, and larval growth rates vary greatly between species. Also, keys are only relevant to a small number of species, the publication of identification keys is based on larval morphology. Sperling et al. (1994) were the first to demonstrate how mtDNA sequencing data from adult specimens of forensically significant flies may be used to distinguish between juvenile members of the same species. Wells and Sperling (1999) discovered using the same methods

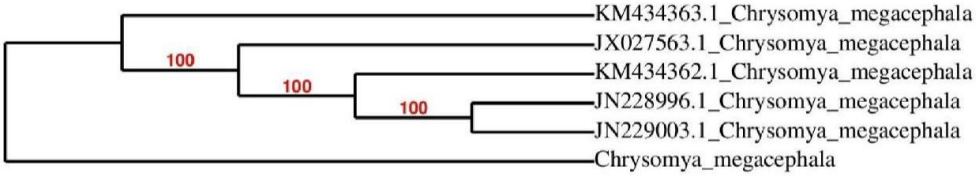

Fig. 1. Phylogenetic tree of *C. megacephala*

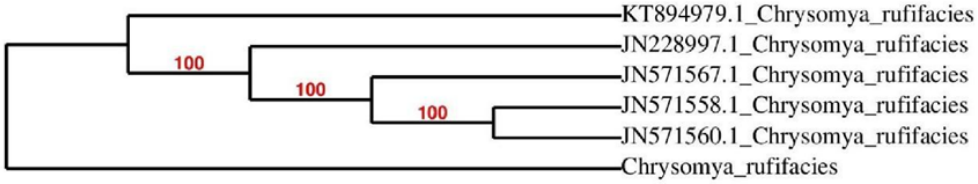

Fig. 2. Phylogenetic tree of *C. rufifacies*

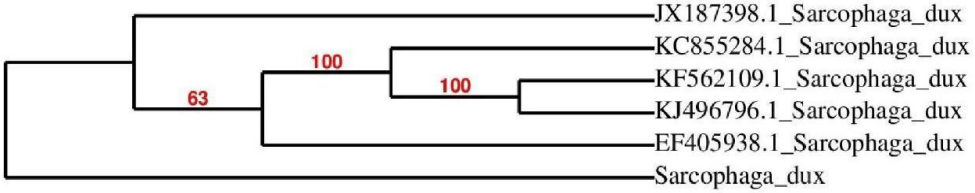

Fig. 3. Phylogenetic tree of *S. dux*

that Calliphoridae species that can be challenging to distinguish taxonomically even in the adult stage have distinctly different mtDNA. Hebert et al. (2003) proposed DNA barcoding, which uses mtDNA to identify unknown using a reference library of categorized specimens (Kress et al., 2005). The mitochondrial COI gene, which is the standard region for DNA barcoding, has the best taxon coverage of all the molecular markers that have been utilized in Diptera (Wells and Stevens, 2008). According to Dawnay et al. (2007), an efficient method for identifying species within the Calliphoridae subfamilies appears to be the examination of the mtDNA. The advantages of mtDNA over nuclear DNA extend beyond this. mtDNA is crucial for molecular identification because it can reveal variations in sequences between closely related species (Waugh, 2007).

This study presents the genetic identification of two Calliphorid species using DNA barcoding. The cytochrome C oxidase subunit I (COI) gene was used to establish the physical differences between two calliphorid species. *Chrysomya's* mean nucleotide frequency distribution was A = 30.4%, T = 38.1%, C = 15.8%, and G = 15.6%, according to Nelson et al. (2007). According to Bajpai et al. (2013), the nucleotide composition was T = 39.6%. A = 31.4%, C = 15.4%, G = 14%. Regional dispersal causes a small but not very noticeable variation in the nucleotide composition. Ullerich and Schöttke (2006) assert that *C. megacephala* and *C. saffranea* share physical and genetic characteristics, but differ ecologically. The physical differences between *C. saffranea* and *C. megacephala* are minimal (Spradbery, 2002), but when species were identified using the COI gene, no interspecific sequences were found, indicating that *C. saffranea* and *C. megacephala* are monophyletic. The highest interspecific sequence variation between *C. saffranea* and *C. megacephala,* according to Harvey et al. (2008) utilizing NO3RD analysis, was only 0.33%, indicating *C. megacephala* and *C. saffranea* species are related. Once more, Harvey et al. (2008) demonstrated that the highest intraspecific variation of *C. saffranea* and *C. megacephala* was 0.18% and 0.34%, respectively, and that the interspecific variance between *C. megacephala* and *C. saffranea* only varied by 0.23%. (Proportion of the 1167 base pairs total for COI) (Abd Al Galil, 2015). His opinion of COI barcoding of *C. megacephala* showed that the nucleotide composition of the genus *Chrysomya* was G = 15%, C = 15.5%, A = 30.85%, and T = 38.3% on average.

The genus Sarcophaga was represented by one member of the Sarcophagidae family, *S.dux*. According to Bajpai and Tewari (2010), the nucleotide composition of all collected Sarcophagidae species was T = 40%, A = 31%, C = 15%, and G = 14%. Meiklejohn et al. (2011) showed that the Australian Sarcophagidae have an A-T bias in their nucleotide composition (mean A = 29.66%, T = 37.02%, C = 17. 43, G = 15.89%). According to Kimura's 2-parameter model, the sequence divergence in the COI barcode region among these sarcophagidae at the species and family levels was 0.0% (K2P). The ranges of sequence divergence at the level of the genus, however, were as follows: Minimum distance = 7.87%, maximum distance = 8.58%, and mean distance. = 8.22%, and standard error = 0.1%. The distribution of sequence divergence, however, was minimum distance = 7.45%, maximum distance = 8.05%, mean distance = 7.75%, and standard error = 0.08%, according to the pairwise distance the distribution of sequence divergence was minimum distance = 7.45%, maximum distance = 8.05 %, mean distance = 7.75% and, the standard error = 0.08%.

The minimum, mean, and maximum distances to the nearest neighbor in the Barcode Gap study of the distribution of distances within each species and the distance to each species' nearest neighbor were 7.45%, 7.55%, and 7.75%, respectively, and the SE was 0.05. According to Bajpai and Tewari (2010), the distance between the two species was 9.4%, but the pairwise difference parameter revealed a distance of 7.45% between the barcode species *S. dux* and its nearest neighbor species *S. ruficornis*. The size difference between *S. peregrine* and *S. ruficornis*, the most closely related species, was 7.75%. The phylogenetic tree revealed that *S. ruficornis* and *S. dux* share the majority of common ancestor features. Out-groups of S. peregrine include *S. ruficornis* and *S. dux*. The Sarcophaga genus had a mean nucleotide composition frequency distribution of G = 15.85%, C = 16.31%, A = 29.99%, and T = 37.84%, according to % Abd Al Galil (2015). A value of 100% matches in this database's BOLD search suggests matches with a high level of confidence, whereas a value of fewer than 99% matches indicates a low level of matching.

The flies collected in this study form the basis for baseline data regarding the collection of local carrion flies and their use in forensic sciences. Molecular analyses suggest the use of DNA barcoding as a practical method for identifying fly species. However, the success of this method depends on the upkeep of a comprehensive and reliable genetic reference catalog in GenBank or other readily available data aggregation sources. Identification

of entomological species, a vital step in forensic investigations, can occasionally be difficult simply based on morphology. By using molecular techniques, species or genera that share a similar morphology can be quickly distinguished and are particularly useful since they can be used at any stage of life. The use of DNA-based sequence data can precisely identify an insect's species, overcoming the drawbacks of morphology-based identification.

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CONFLICT OF INTEREST

No conflict of interest.

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AUTHOR CONTRIBUTION STATEMENT

SB designed and carried out the entire study, and VM supervised it by providing timely guidance. SB analyzed data, and VM assisted in data compilation. SB wrote the manuscript, and VM and VR read, corrected, and approved it.

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