Indian Journal of Entomology Online published Ref. No. e24637 DoI. No.: 10.55446/IJE.2024.1637

MOLECULAR AND MORPHOLOGICAL ANALYSIS OF FORENSICALLY IMPORTANT *SARCOPHAGA ALBICEPS* MEIGEN

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ABSTRACT

Forensic entomology uses necrophagous insects to determine post mortem interval (PMI) in criminal investigations. *Sarcophaga albiceps* Meigen is one among the primary colonisers of dead carcasses and hence its identification is crucial for forensic investigations; *S. albiceps* is a common species of Sarcophagidae found in India. Genitalia of male *S. albiceps* were dissected to identify the species using taxonomic keys and female *S. albiceps* were identified using molecular sequencing. Researchers have adopted a number of strategies like molecular identification, particularly the use of the mtCo1 gene and tools like SEM ultrastructural examinations for identification due to the difficulties in using external morphology. The current study offers baseline information that will allow the researchers and forensic officials to correctly identify *S. albiceps* by morphological, molecular, and SEM examinations.

Key words: *Sarcophaga albiceps*, taxonomy, DNA Barcoding, terminalia, scanning electron microscope, forensic entomology, Mitochondrial COX1 gene, post mortem interval, BLASTn, pregonite, postgonite

Forensic entomology uses the necrophagous insects and other arthropods in medico-criminal investigations (Yan et al., 2023) Carrions attract necrophagous insects, specifically Sarcophagidae, which colonize the decomposing body. Collected flies from the decomposing body can be used in determining the Post Mortem Interval (PMI), which further aids in medio-criminal investigations (Amnedt et al., 2011; Silva et al., 2023). Given the significance of PMI in forensic entomology, accurate identification of species is very importance. Zehner et al. (2004) suggests that the duration of development of each species varies and this in turn affects the estimation of PMI. Sarcophagidae flies well known as flesh flies are prevalent throughout the world, comprising of 171 genera and 3094 species (Pape et al., 2011) and out of them 504 species belonging to 50 genera are reported from India (Nandi, 2002; Sinha and Nandi, 2002a; Sinha and Nandi, 2002b). Even though flesh flies can be collected from animal carcasses, it might be difficult to identify their species due to the ambiguous exterior morphology of these insects which either has a consistent or overly variable features(Talebzadeh, 2020). While it is possible to identify male flies by their genitalia, the most reliable approach for identifying female flies still remains to be molecular sequencing (Fuentes-Lopez et al., 2021). Hence researchers have shown interest in using mitochondrial COI gene for identification of forensic flies owing to the ability to obtain the most proximal result, despite the presence of inter and intra species divergence (Saigusa et al., 2005; Meiklejohn et al., 2011). *Sarcophaga albiceps* is the most common Sarcophagid species in India and it is known to cause ailments such as myiasis and to play a significant part in forensic investigations. Furthermore, *S. albiceps* is cosmopolitan and widespread throughout the country, making it ideal for forensic research. However, the detailed study of flesh flies from the state of Kerala is limited and through the current study we are putting forward the male and female identification of *S. albiceps* using morphological, molecular and SEM studies.

MATERIALS AND METHODS

Flies were collected from Neendakara (8°56'16.8"N 76°32'17.7"E), Kollam, Kerala, India using sweeping nets and ranch fly trap baited with decaying chicken meat to attract flies. The collected specimens were immediately preserved in 70% ethanol for further laboratory studies. The male and female specimens were separated based on their external morphology.The genetalia of male and female *S. albiceps* were dissected out by making incision between 3rd and 4th abdominal segment. The dissected abdominal segments were then subjected to 10% potassium hydroxide (KOH) treatment for 24 hr. After the treatment the segments were taken out, rinsed in distilled water and then the penis, forceps and tergite from the male were carefully separated using sharp blade and needle (Kumar et al., 2021) The separated male genitalia structures were then identified

based on the taxonomic key and description given by Nandi (2002) with the help of Olympus zoom stereo microscope SZ61 and pictures were taken using the attached camera Magcam DC plus 14. The dissected female genitalia were kept aside for SEM analysis. Furthermore, for molecular analysis the metathoracic leg of female *S. albiceps* was cut and preserved in 90% alcohol. Owing to the lack of taxonomic keys for female flies, Sarcophagid females are molecularly identified by targeting the mtCo1 gene. A total of 5 males and 7 females were identified. Genomic DNA was isolated from the tissue in leg using NucleoSpin® Tissue Kit and the quality of the isolated DNA was checked using agarose gel electrophoresis. Furthermore, forward primer LCO (GGTCAACAAATCATAAAGATATTGG) and reverse primer HCO (TAAACTTCAGGGTGA CCAAAAAATCA) were used for PCR amplification. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Post PCR, BigDYe Terminator v3.1 were used for the sequencing. The sequence alignment was carried out using Geneious Pro v5.1 (Folmer et al., 1994; Drummond et al., 2010). For SEM analysis, terminalia of male and female *S. albiceps* were allowed to dry in a glass slide for 24 hr and attached to double stick tape on an aluminum stub. This was followed by a sputter coating with chromium and then viewed under Thermo Scientific Apreo 2 scanning electron microscope.

RESULTS AND DISCUSSION

Sarcophaga albiceps Meigen

Redescription (Figs. 1-5)

Head: Frons is about 3/5 width of one eye; frontal vitta black; Antennae dark brown; parafrontal and parafacial plates black; genal and postgenal areas hairy; proboscis black. Thorax: Grey in appearance with three parallel longitudinal stripes; Acrostichal

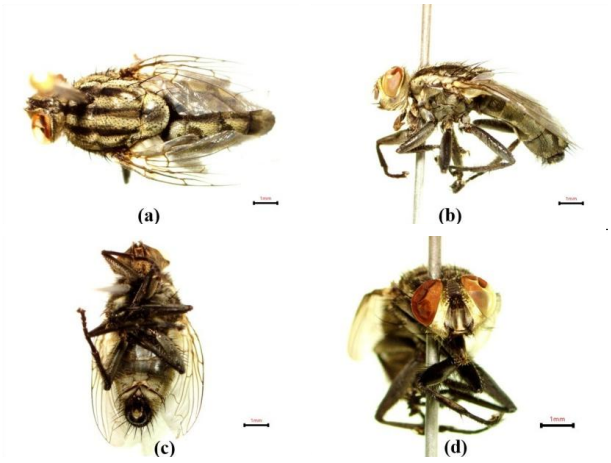

Fig. 1. Adult male–*Sarcophagidae albiceps*- (a) Top view; (b) Lateral view; (c) Ventral view; (d) Front view

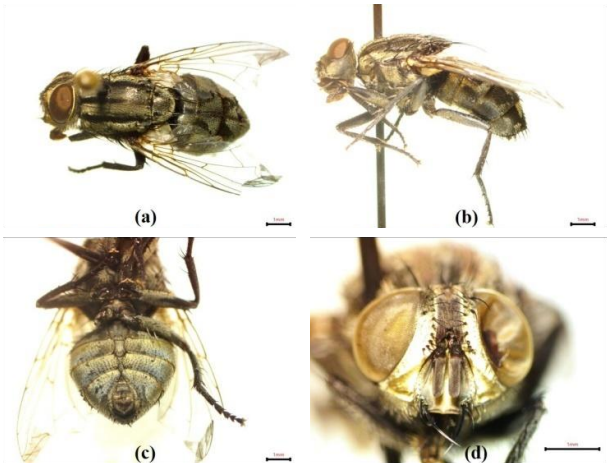

Fig. 2. Adult female–*Sarcophagidae albiceps*- (a) Top view; (b) Lateral view; (c) Ventral view; (d) Front view

Fig. 3. *Sarcophaga albiceps* –male genitalia (a) forceps; (b) 5th sternite; (c) penis in lateral view (d) penis in ventral view

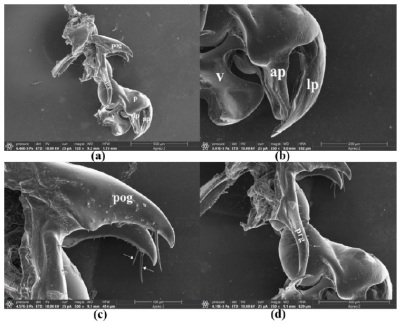

Fig. 4. SEM photographs – *S. albiceps* male terminalia (a) penis; (b) enlarged view of ventralia; (c) enlarged view of postgonite; (d) enlarged view of pregonite. Abbreviations: pog- postgonite, prg- pregonite, p- paraphallus, ap- apical paraphallus, lp- lateral paraphallus

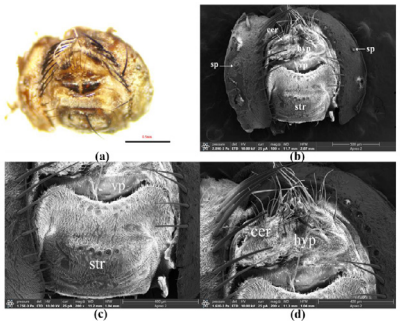

Fig. 5. SEM photographs– *S. albiceps* female terminalia (a) microscopic image; (b) SEM view; (c & d) enlarged view of sternite and cercus. Abbreviations: Sp- spiracle, vp-vaginal plate, hyp- hypoproct, cer-cercus, str- sternite

bristles 0+1; dorsocentral bristles 5+5; Inter-alar 1+3; Post sutural 1; Humeral 3; post humeral 2; post-alar 2; supra-alar 4; katepisternal 1+1+1; mesopleural 6; hypopleural 8. Abdomen: Abdomen blackish with silver checkered pattern; median marginals bristles on 2nd and 3rd abdominal tergites absent but 2nd abdominal segment with 2 and 3rd abdominal segment with 2-3 well developed lateral marginal bristles. Absence of marginal bristles on second and third abdominal tergites; Presence of 2 marginal bristle on second tergite and 2-3 marginal bristle on the third abdominal tergite; 4th tergite has a pair of well-developed stout lateral marginal bristles; 5th sternite Y- shaped. Genitalia: The dissected genitalia structure consist of a penis, tergite and sternite (Fig. 3). First and second genital segment black in colour; absence of marginal bristles on the second segment; inner forceps elongated and presence of spines at the inner surface of sub-apical part with bunch of hairs on the basal region; kidney shaped outer forceps with hairs on the distal half; middle portion of the anterior paramere curved while the posterior paramere flat; presence of tuft of hairs on the apex region of posterior paramere; theca and paraphallus long, curved and pointed at end; styli of glans short; Anterior portion of ventralia curved.

In male morphological identification is done with -(i) head, thorax and abdomen (ii) confirmation by genital structures. They share similar external morphology features with closely related species such as *S. albiceps*, *Parasarcophaga serice* Walker and *Parasarcophaga hirtipes* Wiedemann. However, there is a clear difference in the male genital structures. Through these male terminalia differences, structure of ventralia and styli of glans in this case, the aforementioned species can be identified (Nandi, 2002). This is unlike other families such as Calliphoridae where taxonomic features of head, thorax and abdomen can be used (Ji et al., 2021). Sharma et al. (2017) showed the morphological features of head, thorax, abdomen and genitalia structure in order to identify *S. albiceps*. A new species *Sarcophaga* (*Liosarcophaga*) *geetai* Algalil and Zambare, 2016 was identified in India, where the male species was described using morphology and terminalia (Abd Algalil and Zambare, 2016). Giroux et al. (2010) carried out a study using the male genitalia features especially the phallus region. This study also focused on morphology and terminalia for male *S. albiceps*.

Molecular analysis: The sequence data obtained through the analysis is as follows. 560 bp DNA, cytochrome c oxidase subunit 1 (Accession No. OR142122.

BLASTn analysis showed 100% similarity with mtCo1 of *S. albiceps* in the GenBank sequences with accession number MK240350.1. The similar sequences available were used to create a phylogenetic tree (Fig. 6). Maximum Likelihood method and Tamura-Nei model were used to find the evolutionary history (Kimura, 1980). Automatically applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Tamura-Nei model, and then choosing the topology with the highest log likelihood value, the initial tree for the heuristic search was created. A total of 11 nucleotide sequences were taken for analysis in which their first, second, third and non-coding codon positions were included. This was followed by evolutionary analyses in MEGA11 (Kumar et al., 2018). Between sequences, the number of base substitutions per site is displayed. A bootstrap approach (500 repetitions) was used to get the standard error estimate (s) that are displayed above the diagonal. The Maximum Composite Likelihood model was used for analysis. There were 11 nucleotide sequences in this investigation. Codon positions 1st+2nd+3rd+Noncoding were included. For each set of sequences, all ambiguous locations were eliminated (pairwise deletion option). The final dataset contained 636 locations altogether. In MEGA11, evolutionary analyses were carried out (Kumar et al. 2018). Zehner et al., (2004) has carried out studies using 12 species of Sarcophagidae flies and demonstrated a bootstrap value of about 90% in the neighbor joining tree. Similarly, this study done with bootstrap analysis with 500 replicates (Fig. 6).

Morphological identification of some female dipterans is difficult and in case of female sarcophagids, it is nearly impossible since there are high similarities

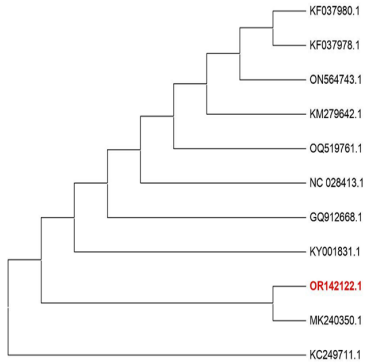

Fig. 6. The GenBank data of submitted species showing maximum similarity with accession number along with the newly generated accession number from the current study confirming the *S. albiceps*

in external morphology. Hence, the challenge of identifying female flies was earlier tackled by collecting copulating pairs and identifying the male (Ekrem et al. 2010; Zhang et al. 2013). mtCo1 is gene frequently used for species identification (Achint and Singh, 2021; Ghosh et al.2022). Tan et al. (2010) used mitochondrial COI gene to identify flesh fly species like *Sarcophaga dux* Thomson*, S. albiceps* Meigen *S. princeps* wiedemann and *S. ruficornis* F, mtCo1 was used to identify *Sarcophaga argyrostoma* (Robineau-Desvoidy), *S. albiceps* (Meigen) and *Wohlfahrtia nuba* (Wiedemann) (Aly et al., 2013); Guo et al. (2011) identified *S. dux* through molecular analysis Meiklejohn et al. (2011) identified *S. dux* through mtCo1. There are limitations such as the availability of DNA barcode data and heteroplasmy (presence of several mitochondrial haplotypes in single individual) (Ekrem et al., 2007; Song et al., 2008; Szyp-Borowska and Sikora, 2019). Along with that, factors like Interspecific hybridization and paraphyly among different insect taxa is also contribute to the inaccuracy of DNA barcoding. (Funk and Omland, 2003; Virgilio et al., 2010).

The SEM study done by Chaiwong et al. (2007) revealed the presence of sensillae in male terminalia of *Parasarcophaga* (*Liosarcophaga*) *dux* Thomson. The postgonite region possess single long sensillae and a group of short sensillae. Furthermore, cerci also possesses sensillae. SEM analysis of male genital region of *Sarcophaga* (*Liosarcophaga*) *dux* was done Sukontason et al. (2014) and structures like pregonite, postgonite, styli of glans showed intricacies which can be perhaps standardized for taxonomic keys. The present study includes SEM analysis to understand the ultrastructure of genitalia of both male and female *S. albiceps*. Ultrastructure images of the penis showed bristles and hairs which are more evident. Apical region of the postgonite has two hairs in which one is short and other is long. In addition, pregonite also comprises minute hairs arranged from the base to the apex (Fig. 5). The female genital part images given in Fig. 6 cerci region with abundant minute hairs, presence of single spiracle on either side of the tergite. Sternite possess 5 bristles, vaginal plate is sclerotized but no conspicuous, cercus is arranged to sideways with a gap in between, hypoproct is having few upstanding hairs and abundant minute hairs which gives it a membrane kind of appearance.

In forensic entomology, it is crucial to correctly identify necrophagous insects. *S. albiceps* is one among the species that colonize dead carcasses and their identification is even more crucial in forensic investigations. Researchers have adopted a number of strategies for the identification due to the difficulties in using external morphology, including molecular identification, particularly mtCo1 can be used along with SEM ultrastructural examinations.

ACKNOWLEDGEMENTS

Authors thank the CHRIST (Deemed to be University), Bangalore for providing the support for carrying out the research. In addition, authors also thankful to Kerala Forest Research Institute (KFRI), Peechi and Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram for providing the support for Microscopic images and Molecular sequencing. Authors are grateful to Dr. Madhu Bala and Rohit Kumar (Punjabi Patiala University) for the help in species identification.

FINANCIAL SUPPORT

No funding received.

AUTHOR CONTRIBUTION STATEMENT

BMN designed, executed the work, acquired the data and drafted the manuscript. MT supervised, orchestrated data analysis and collaborated in drafting the manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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(Manuscript Received: September, 2023; Revised: March, 2024;
Accepted: March, 2024; Online Published: April, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24637