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SEM analysis of a forensically important puparia †

Vitthal More and Bansode Sarika *

University, Aurangabad, India; vitthal67l@gmail.com

* Correspondence: sarikaabansode@gmail.com;

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Abstract: Use of insects in forensic science needs a correct identification of insects to correlate their decomposition rate with post mortem interval. This study emphasizes on puparia based identification of *Chrysomya megacephala* with *C. rufifacies* of a road cadaver of a dog found at Osmanabad district, Maharashtra, India. Scanning electron micrographs of both species were studied to differentiate them. Distinguishing characteristics of both species observed during present study are pattern of folding in frontal field, number of anterior spiracles, posterior spiracles, number of tubercles, and structure of button, spiracular hair and middle sacrum. This study provides a comprehensive key to identify fly species using scanning electron micrographs of puparium.

Keywords: Forensic, entomology, puparia, cadaver, calliphoridae

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1. Introduction

Arthropods are considered the most diverse group on the planet. Since ancient times blow flies have been known to the people [1]. Dipteran flies are very important in the forensic field to calculate elapsed time since death in humans as well as animals [2]. To estimate elapsed time since death knowledge of insect's development is very important. [3]. Families important in forensic entomology are Calliphoridae, Sarcophagidae and Muscidae, Silphidae, Staphylinidae, Cleridae and Dermestidae [4, 5]. Commonly known blow fly *Chrysomya megacephala* (Fabricius, 1794) and Australian hairy maggot blow fly are reported as myiasis causing agents in humans as well as animals [6]. *C. megacephala* an oriental latrine fly also known to transmit various pathogens like *Salmonella* and *Shigella* [3, 6]. Though *C. megacephala* has global distribution and association with humans as a myiasis causing agent [8] it also plays an important role for humans as adults of this fly are pollinators of many fruits [9]. *Chrysomya rufifacies* (Macquart, 1842) is an Australian blowfly. This Calliphoridae species is one of the important hairy blowflies. Morphology based identification of fly puparium is difficult due to similarities in structure. To overcome this problem many techniques have been used to identify fly species using light microscope and scanning electron microscope (SEM) [10- 12]. Generally rarely puparia are being considered in the identification of the flies. Puparium retains maximum characters present in the third instar hence can be used as an important tool in forensic cases [13]. Most of the time rearing immature insects to an adult stage is a very time consuming process and may lead to delay in the investigation. So scanning electron microscopy of the puparium is performed to identify the fly species. Micrographs of various parts of the puparium to distinguish two Calliphoridae namely *C. megacephala* and *C. rufifacies* are presented here.

2. Materials and Methods:

Present study carried out in Osmanabad district of Maharashtra, India. A cadaver of a dog was observed on Osmanabad highway beside the road. Body of a dog was almost decomposed and clusters of pupa were observed next to it (figs a-b). Collection of pupa

was done for the species identification and samples brought to the laboratory. As a dog cadaver was completely decomposed, larvae were absent in the surrounding area and only puparіums were observed there. Some puparіums were kept in the cage for adult emergence. Scanning electron microscopy was performed to study fly species associated with the cadaver. It was quite difficult to select specific pupa for scanning electron microscopy from clusters of pupa. Firstly dissimilar pupa identified using compound microscope and then proceed for scanning electron microscopy. Puparia placed in hot water to arrest their further development process. Puparia cleaned by placing them in a vial with warm soapy water and shaking the vial softly in an attempt to get rid adhering debris on the puparial surface. A small sized paint brush used to brush pupa in order to remove soil particles. All pupa were washed in distilled water until no soapiness was observed. Finally, the pupa is attached to double-stick tape on aluminum stubs in order to coat with gold in the sputter-coating apparatus to enable viewing a JEOL-JSM840A scanning electron microscope.

3. Results and Observations:

In pupation, the cuticle of larva becomes highly sclerotized and contracts longitudinal musculature and forms the pupa. During sclerotization pupa changes its color from deep pink to dark brown. Generally both the puparia were oblong shaped (figs c-d). Unique pattern of frontal field was observed in SEM of both puparia (figs e,f). Structure of the mouth scar was very prominent and distinct in the SEM of *C. rufifacies* (fig h) whereas such a mouth scar didn't show a marked deep cavity in *C. megacephala* (fig g). Structural folds observed at the frontal field of *C. rufifacies* such as structural folds were absent in *C. megacephala*. SEM observations of *C. megacephala* and *C. rufifacies* revealed that both species have marked differences in their puparia. Texture of integument of puparia was smooth in *C. megacephala* but in *C. rufifacies* it was with numerous pointed spines. Spines can be observed by naked eyes in a puparium (figs c, d). Pair of anterior spiracles were located laterally near the prothorax in both puparia. Twelve anterior spiracles were observed in *C. megacephala* (fig k) and 10 in *C. rufifacies* (fig n). Ten tubercles found very distinct in *C. rufifacies* (fig j). SEM of posterior spiracles . *megacephala* showed a pair of posterior spiracular discs containing three straight slits and a relatively thin and incomplete peritreme and an indistinct button-like structure in the open area of the peritreme (fig k). Spiracular disc interspaced with the bundles of relatively thin and multibranched spiracular hair. SEM of posterior spiracles *C. rufifacies* showed the prominent button like structure and a complete peritreme that stabilizes the margin of a posterior spiracle. The elongated tubercles encircling body segments were located along the body and were slender in shape, with a tip having circular rows of spines. Each spiracular disc contained three straight slits and a relatively thick peritreme (fig l).

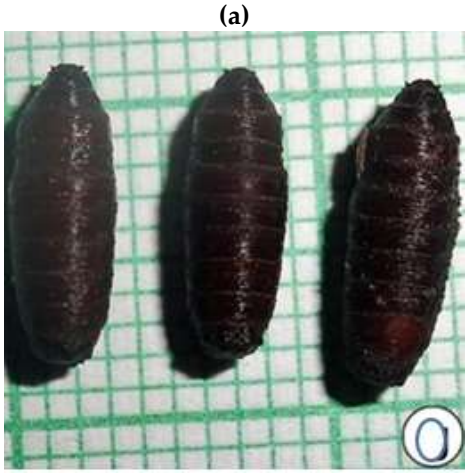
SEM of posterior spiracles *C. megacephala* and *C. rufifacies* (figs k, l) have shown the inner-spiracular hair cluster (ishc) was associated with the outer margin of the inner spiracular opening (i so). The middle inner spiracular hair cluster (mi shc) was located between the inner and middle spiracular openings, attached to the intermediate structure (ims). The middle-outer spiracular hair cluster (moshc) was associated to the outer margin of the middle spiracular opening (m so) and the outer spiracular hair cluster (o shc) was associated to the outer margin of the outer spiracular opening (o so). The position of the spiracular hair in relation to the spiracular openings, the attachment area it occupied and the extent of the branching of the spiracular hairs considered for diagnostic purposes.

Key for identification of *Chrysomya megacephala* and *Chrysomya rufifacies* puparia based on SEM images

1. Numerous slender spines at the tip of tubercles. 2. Anterior spiracle with 9-11 lobes fig n. 3. Posterior spiracular peritreme complete and relatively thick. 4. 10 papillae fig j. 5. Prominent button/ spiracular bud like structure and a complete peritreme that stabilizes the margin of a posterior spiracle fig l. 6. Unique structural folds at frontal field with specific patterns like hairpin fig h. 7. Distinct mouth scar with remarkable deep cavity fig h.	*Chrysomya rufifacies*
1. Absence of numerous slender spines at the tip of tubercles. 2. Anterior spiracle with 10-13 lobes fig m. 3. Posterior spiracular peritreme incomplete and relatively thin. 4. 3 pairs of papillae fig i. 5. Prominent indistinct button/ spiracular bud like structure and an incomplete peritreme that stabilizes the margin of a posterior spiracle fig k. 6. Wrinkled frontal field. Fig f, g. 7. Absence of distinct mouth scar with remarkable deep cavity fig g.	*Chrysomya megacephala*

(a)

(b)



(c)

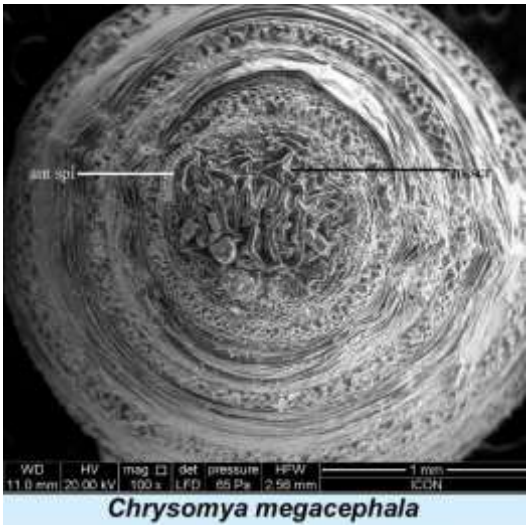
(d)


Chrysomya megacephala

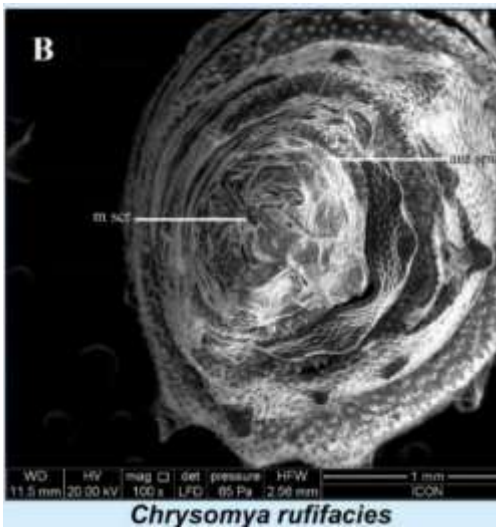
(e)


Chrysomya rufifacies

(f)


ant spi
m sct
Chrysomya megacephala

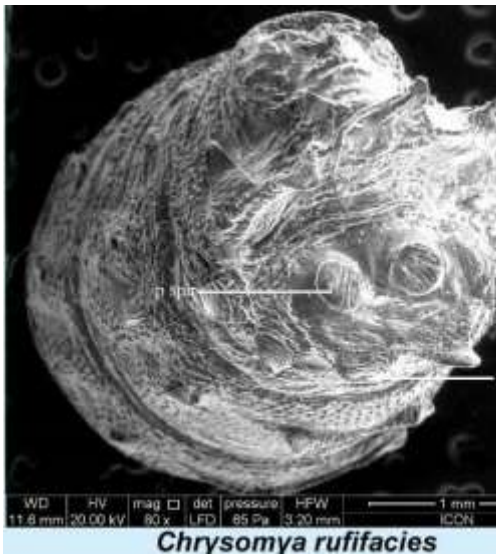
(g)


B
m sct
Chrysomya rufifacies

(h)


p spir
p tub
Chrysomya megacephala

(i)


p spir
Chrysomya rufifacies

(j)

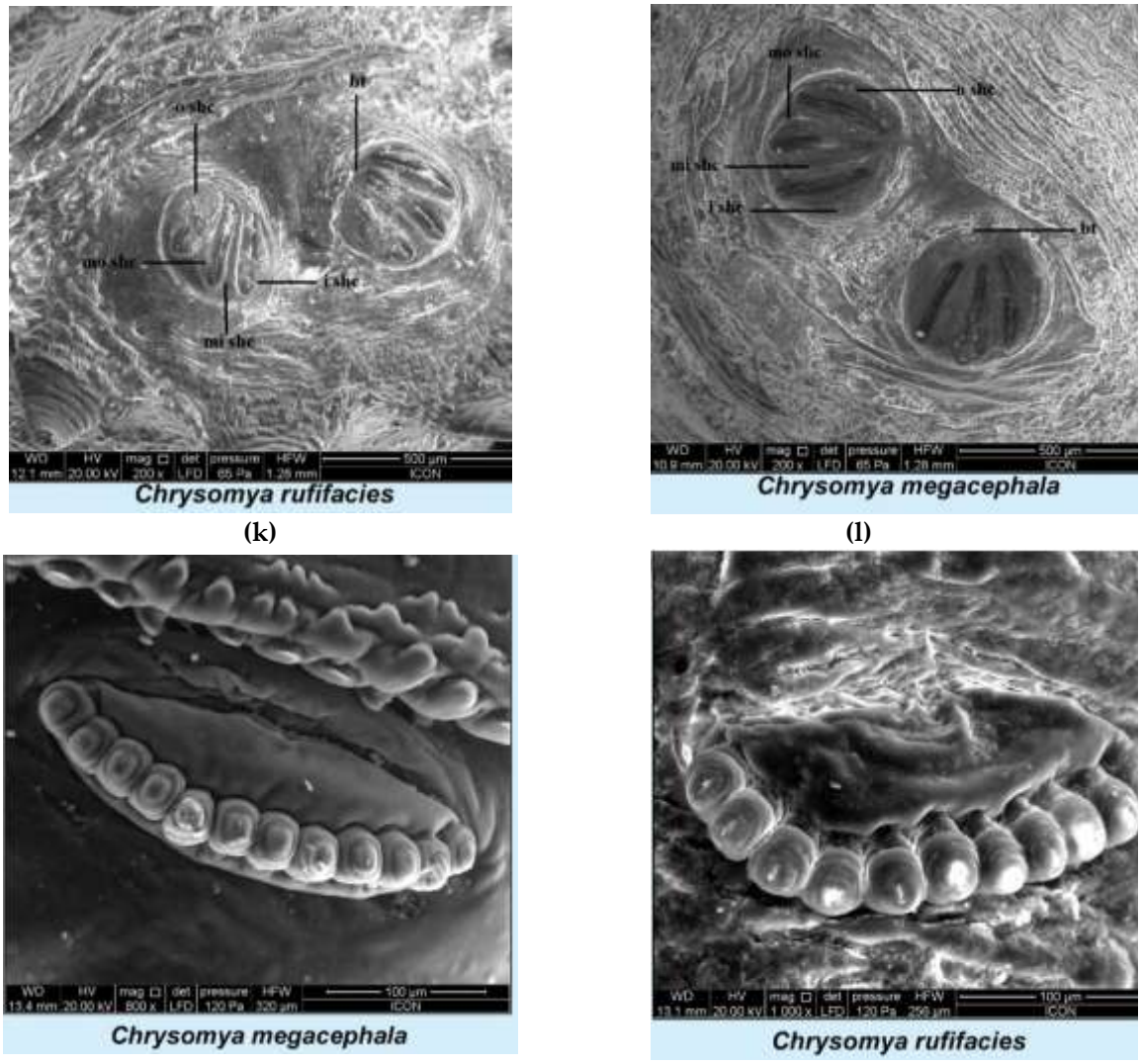


Figure 1. a- Decomposed skeleton of a dog cadaver, **b**-cluster of puparia, **c**- puparium of *C. megacephala*-**d**- puparium of *C. rufifacies,* **Scanning electron micrographs of puparia: e-f**- frontal field of puparia, **g-h**-frontal field of puparia showing mouth scar (m scr) and anterior spiracles(ant spi), **i-j**- SEM showing posterior spiracles and tubercles,(p spir- posterior spiracle, p tub- posterior tubercles).**k-l**- SEM showing structure of posterior spiracles , (innerspiracular hair cluster (ishc), middle inner spiracular hair cluster (mi shc), middle-outer spiracular hair cluster (moshc), spiracular hair cluster (o shc),bt- button, **m-n**- anterior spiracles).

4. Discussion

Scanning electron micrographs of *C. megacephala* and *C. rufifacies* have been presented as a key for identification. This study assures the identification of these two forensically important fly species by using puparia. Previously very few scientists have emphasized on puparial identification so present study may provide a new reckoner for the researchers.

During pupariation and pupation post feeding larvae shrinks, integument becomes hardened. Integuments of the last larval instar transform into puparia and all diagnostic characteristics of third instar are present in puparia. Some characters that remain unchanged are cephalopharyngeal skeleton, anterior spiracles, posterior spiracles, intersegmental spines, button etc. Unique folding patterns of the anterior field are due to retraction of psedocephalon [14]. The unique folding patterns of the anterior field in puparia are identified for its diagnostic potential in differentiating species and are used for the first time during the current study.

Previously *C. megacephala* has been studied by using SEM by [15-16]. They have studied the third instar larva to observe its shape, body segments, dorsal organs, terminal organs, ventral organs and mouth hooks. More emphasis was given on anterior and posterior spiracles for the identification. Ten to thirteen papillae were observed in *C. megacephala* which correlates with this study. During present study 12 papillae were observed on the anterior surface of puparia of C. *megacephala* and 10 papillae on the *C. rufifacies*. Number and structure of the tubercle is another important characteristic presented during present study. Tubercles of *C. megacephala* and *C. rufifacies* were completely different from each other. SEM of *C. rufifacies* showed 10 very distinct tubercles, pointed and having plenty of hair like structures. Tubercles of *C. megacephala* were quite small. This study resembles the result presented by [17] who distinguished Calliphoridae and Sarcophagidae fly species based on number of tubercles and presence of numerous small spines at the tip. Presence of tubercle also has been used to differentiate the species *C. rufifacies* and *C. villeneuvi* [10].

Unique pattern of formation of the frontal field was observed in both the species. Retraction of pseudo cephalon leads to formation of an aperture called mouth scar (m scr). *C. rufifacies* showed very distinct mouth scar with remarkable deep cavity such a mouth scar was absent in *C. megacephala*. Structural folds were observed at the frontal field of *C. rufifacies* puparium. Such structural folds were absent in *C. megacephala*. Number of anterior spiracles did not show any variation. Similar study was done by [16] to differentiate *C. chloropyga* and *C. putoria* based on the differences in frontal field of puparia. Texture of the puparium surface of *C. megacephala* was smooth as compared to *C. rufifacies*. Small spine-like structure observed all over the puparia of *C. rufifacies* which is a very unique feature. One can efficiently use this structure to differentiate *C. megacephala* and *C. rufifacies*. [17] have performed morphological comparison between the puparia of *C. rufifacies* and *C. villeneuve* using SEM. Their study also emphasized on structure of tubercles, spines present on the body segment, number of papillae and number of anterior spiracles.

Sukontason et al. have studied morphological comparison of *C. megacephala* puparia with other blowflies including (*Chrysomya nigripes* (Aubertin), *Chrysomya rufifacies* (Macquart), *Chrysomya villeneuve* (Patton), *Lucilia cuprina* (Wiedemann), and *Hemipyrellia ligurriens* (Wiedemann)) and a housefly (*Musca domestica* L.) [18]. Their findings revealed that the features of anterior ends and the profiles of the posterior spiracles, structure and arrangement of spiracular disc, spiracular slits had markedly distinguishing characteristics. Morphometric analysis of the length and width of the puparia, along with the length of the gaps between the posterior spiracles of seven fly species, displayed differences among them. This provides a key to identifying the puparia of these seven fly species.

5. Conclusion:

This study is the first attempt of this type in Maharashtra and provides ample information to differentiate fly species based on SEM of puparia. Instead of rearing immature stages to an adult, forensic entomologists can identify species based on puparia. It not only saves time but also leads to a new reckoner of species identification.

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