A PRELIMINARY GEOMETRIC MORPHOMETRICS ASSESSMENT OF TWO FORENSICALLY IMPORTANT BLOW FLY LARVAE IN MALAYSIA, *Chrysomya megacephala* (Fabricius) AND *Chrysomya rufifacies* (Macquart) (DIPTERA: CALLIPHORIDAE)

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ABSTRACT

In forensic entomology, the age of blow fly larvae (Diptera: Calliphoridae) that feed on decomposing human tissues can be used as reference in minimum post mortem interval (mPMI) estimation. To establish mPMI based on larval age, it is important to correctly identify larva species based on their morphological characteristics as larval developments from where they were collected are species-specific. Recently, landmark-based geometric morphometric analysis has been found useful to discriminate species and provide visual shape variations. The objective of this study was to assess the utilization of this technique on two forensically important blow fly species in Malaysia, i.e. the *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) based on the cephalopharyngeal skeletons of the larvae. A total of 10 landmarks on cephalopharyngeal skeleton were established and analyzed with geometric morphometric functions in MorphoJ™ software. Cephalopharyngeal skeleton centroid size, which represented the individual cephalopharyngeal skeleton shape profile, were statistically different between *C. megacephala* and *C. rufifacies* (p<0.001). Based on the landmark plot shifts, the two species could be differentiated based of parastomal bar, the clipeal arc, apical hook, upper margin of ventral cornu and lower margin of ventral cornu. These differences were well defined in visual presentation by using principal component analysis with 100% cross validation reassignment percentage. However, large scale study should be considered for a more complete cephalopharyngeal skeleton shapes profiles of forensically important Calliphoridae.

Keywords: Cephalopharyngeal skeleton, geometric morphometric, morphological landmarks, forensic entomology, Calliphoridae

ABSTRAK

Dalam bidang entomologi forensik, usia larva langau (Diptera: Calliphoridae) yang memakan tisu reput manusia dapat digunakan sebagai rujukan untuk menganggar selang masa pasca kematian minimum (mPMI). Dalam penentuan mPMI berdasarkan usia larva, pengenalpastian spesies serangga berdasarkan sifat morfologi adalah penting kerana kadar perkembangan larva dari tempat ia dikutip adalah khusus mengikut spesies. Terkini, analisis geometri morfometri berdasarkan plot mercu tanda didapati berguna untuk membandingkan spesies dan menunjukkan variasi bentuk secara visual. Objektif kajian ini adalah untuk menilai keberkesanan penggunaan teknik ini terhadap dua spesies lalat berkepentingan forensik di Malaysia iaitu *Chrysomya megacephala* (Fabricius) dan *Chrysomya rufifacies* (Macquart) berpandukan rangka sefalofarinks larva. Sebanyak 10 mercu tanda pada rangka sefalofarinks telah dipilih dan dianalisis menggunakan fungsi geometri morfometri dalam perisian MorphoJ™. Profil bentuk rangka sefalofarinks ditunjukkan melalui nilai saiz sentroid dan mendapati wujudnya perbezaan signifikan di antara *C. megacephala* dan *C. rufifacies* (p<0.001). Berdasarkan pergerakan plot mercu tanda, kedua-dua spesies dapat dibezakan melalui bar parastomal, arca klipeal, cangkuk apikal, margin atas kornu ventral dan margin bawah kornu ventral. Perbezaan ini dapat dijelaskan melalui perbandingan visual dalam analisis komponen utama dengan 100% pengklasifikasian semula validasi silang. Namun, kajian berskala lebih besar perlu diertimbangkan bagi mendapatkan profil rangka sefalofarinks larva Calliphoridae berkepentingan forensic yang lebih lengkap.

Kata kunci: Rangka sefalofarinks, geometri morfometri, mercu tanda morfologi, entomologi forensik, Calliphoridae

INTRODUCTION

Chrysomya megacephala (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) are the two dominant blow fly species representing forensic entomofauna in Malaysia (Lee et al. 2004; Nazni et al. 2015). The immature stage development of these species has been studied in laboratory for the purpose of minimum post mortem interval (mPMI) estimation in death investigations (Ahmad Firdaus et al. 2009; Thevan et al. 2010) and they have been used as laboratory subjects in research related to forensic entomology practice (Rumiza et al. 2008; Rosilawati et al. 2014). In many forensic cases in Malaysia and neighboring regions, *C. megacephala* and *C. rufifacies* were the primary indicators to assist mPMI estimation (Sukontason et al. 2001; Sukontason et al. 2008; Kumara et al. 2012).

The larval stage of these two species can be distinguished based on their morphological features which are displayed by their external appearances, conditions of the posterior spiracles and shape variations of their mouthparts or cephalopharyngeal skeletons (Ishijima 1967; Sukontason et al. 2004). In the immature stage of blow fly, the cephalopharyngeal skeleton is invaginating mouthparts in the cephalic region of the larva, consisting of pharyngeal sclerites and mandibles to facilitate food intake (Romoser 1981; Teskey 1981). Recently, these structures have been suggested as alternative growth parameters to larval body length for mPMI estimation (Rabbani & Zuha 2017; Eliza & Zuha 2018).

In cases where the larvae are improperly preserved, cephalopharyngeal skeleton might be the only diagnostic part available for identification. Species identification will be more difficult as reference to dichotomous taxonomic keys requires combined knowledge of

cephalopharyngeal skeleton shape characteristics and other larval features. Therefore, geometric morphometric analysis can be considered as an appropriate application to provide shape profile of the cephalopharyngeal skeleton.

Geometric morphometric analysis has been recently utilized as practical solution to visualize variations in biological shapes (Dujardin 2008; Webster & David Sheets 2010; Zelditch et al. 2012; Tatsuta et al. 2018). Apart from its extensive use in anthropology (Bookstein et al. 1999), similar approach has been used to discriminate dipteran species and establish phenetic relationship in insects including those of forensically important species (Hall et al. 2014; Nuñez-Rodríguez & Liria 2017; Sontigun et al. 2017). Recently, its application has been extended to immature stage of forensic blow flies based on cephalopharyngeal skeleton shapes (Nuñez & Liria 2016). Considering this recent development in forensic entomology, the objective of this research was to provide baseline data of cephalopharyngeal skeleton shape profiles and determine shape variations between *C. megacephala* and *C. rufifacies* from Malaysia.

METHODOLOGY

Sample Preparation

Between July and August 2018, *C. megacephala* and *C. rufifacies* were collected from rabbit carcasses placed in an open environment at Forensic Science Simulation Site, Universiti Kebangsaan Malaysia, Bangi. Larvae were mainly consisting of largest third instar in homogenous size were killed in near-boiling water (≈80º C) for 30-40 seconds and preserved in universal glass vials containing 70% ethanol (Amendt et al. 2007).

In the laboratory, larvae were immersed in 10% KOH solution for 24 hours. Cephalopharyngeal skeleton was carefully separated from the larval body, with the gut contents and adhering tissues removed in 10% KOH. Subsequently, the cephalopharyngeal skeleton was soaked in 10% acetic acid and 70% ethanol for 10 minutes each. Cephalopharyngeal skeleton was then mounted on glass slide in lateral position with Berlese Fluid and a 5 mm rounded coverslip (Eliza & Zuha 2018).

Cephalopharyngeal Skeleton Landmark Acquisition and Data Analysis

Images of cephalopharyngeal skeleton were captured by using an SMZ745T stereomicroscope (Nikon, Japan) fitted with microscope USB2.0 CMOS camera (Toupcam, China). The images were converted to a readable format using tpsUtil (Version 1.74) and landmarks were plotted by using tpsDig2 (Version 2.31). The 10 landmarks were chosen based on Nuñez and Liria (2016) with modification (Fig. 1). Geometric morphometric analysis of cephalopharyngeal skeleton was carried out by using MorphoJ™ software (Klingenberg 2011), which includes visualization of thin-plate spline transformation grid and principal component analysis. Centroid sizes were classified based on species as independent group and analyzed by using independent sample *t*-test (α=0.05) in SPSS™ Version 22.

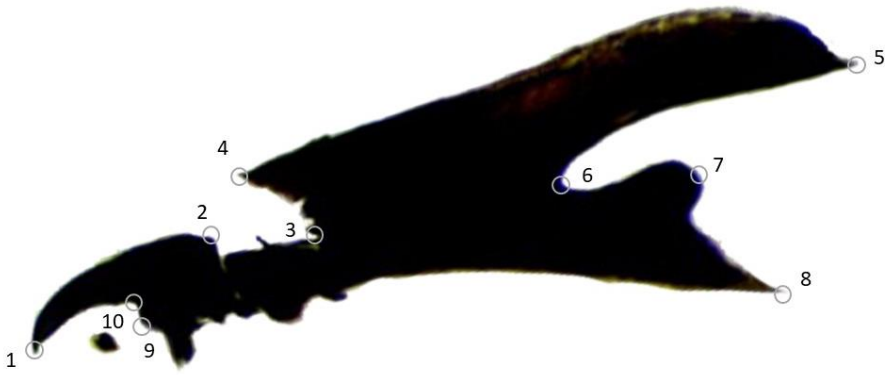


Figure 1 Geometric morphometric landmark plots on *C. megacephala* third instar larva after Nuñez-Rodríguez & Liria (2017) with modification. 1. Apical hook, 2. Dorsal apodeme of mouth hook, 3. Base of parastomal bar, 4. Clipeal arc, 5. Dorsal cornu, 6. Concavity of pharyngeal sclerite, 7. Upper margin of ventral cornu, 8. Lower margin of ventral cornu, 9. Ventral apodeme of mouth hook, 10. Basal hook

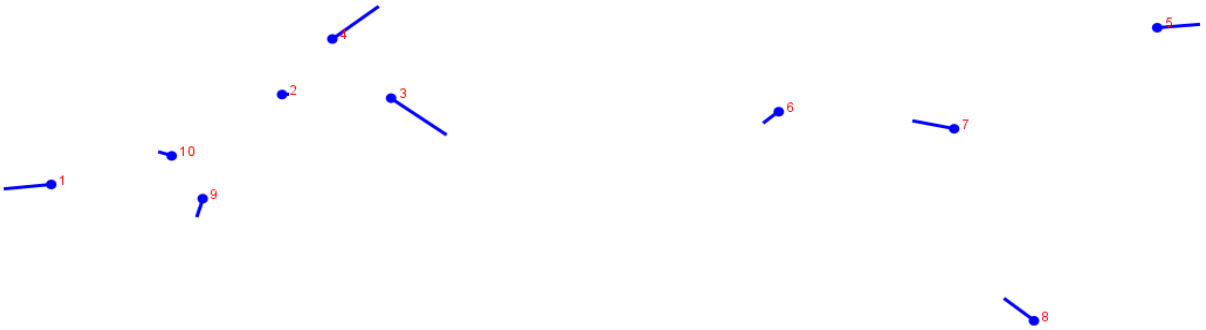
RESULTS

Centroid Size Comparison

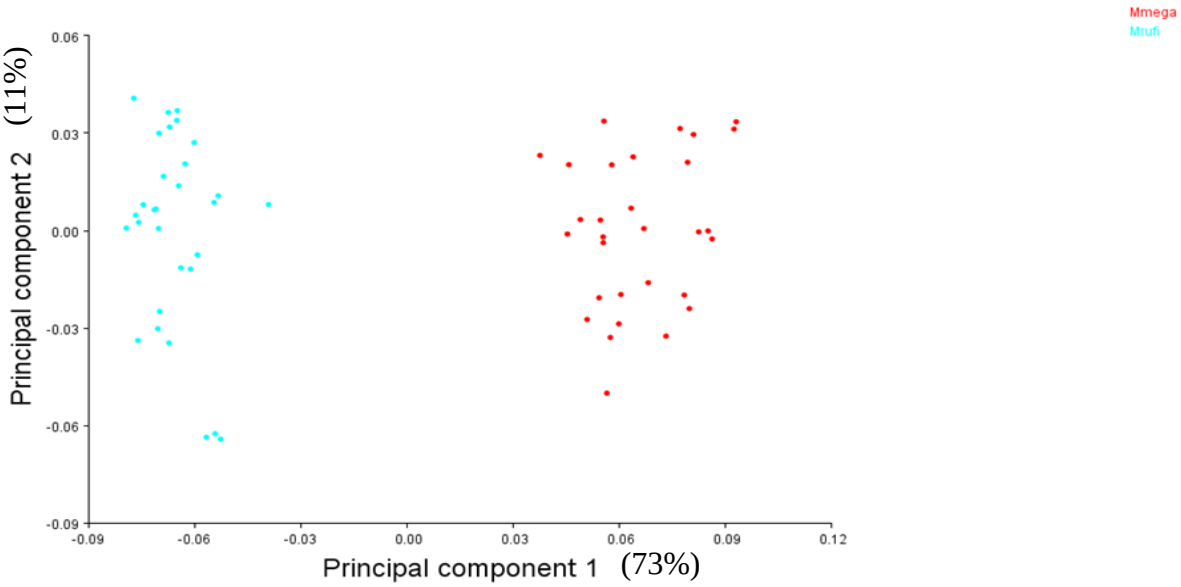
Mean centroid size of *C. megacephala* (2.049±0.084) was significantly different from *C. rufifacies* (2.117±0.075), t (30) =-3.29, p<0.001, d=0.85 (large effect size), which indicates distinction of shapes between the two species.

Cephalopharyngeal Skeleton Landmark Dispositions

Figure 2A shows the landmark shifts in *C. megacephala* and *C. rufifacies*. Disposition of landmarks was more apparent in landmark 3 (base of parastomal bar), 4 (the clipeal arc), followed by landmark 1 (apical hook), 7 (upper ventral cornu) and 8 (lower ventral cornu) (Fig. 2A). Figure 2B shows visual discrimination of *C. megacephala* and *C. rufifacies* with the first two principal components accounted for 73% and 11% respectively. Mahalanobis distance obtained by pairwise comparisons between the two species (15.9587) showed highly significant differences (permutation 1000 rounds in MorphoJ: p<0.0001). Proscrustes disctances (0.1310) also showed highly significant differences between *C. megacephala* and *C. rufifacies* (permutation 1000 rounds in MorphoJ: p<0.0001). The reclassification based on cross validation test revealed 100% correct group assignments.



A



B

Figure 2 A. "Lollipop" diagram showing landmark shifts in *C. megacephala* and *C. rufifacies* cephalopharyngeal skeleton. B. Variations in cephalopharyngeal skeleton shape of *C. megacephala* and *C. rufifacies* based on principal component analysis

DISCUSSION

Geometric morphometric analysis was found to be useful to assess and discriminate the shape profiles of *C. megacephala* and *C. rufifacies* cephalopharyngeal skeletons. Based on the landmark shifts, both species can be differentiated based on parastomal bar base, the clipeal arc, apical hook, upper ventral cornu and lower ventral cornu. These variations were in conformity with the descriptions by Ishijima (1967) on the morphological differences in the apical hook, dorsal cornua and ventral cornua in both species. Nuñez-Rodríguez and Liria (2017) made the comparison between *C. megacephala* and *Chrysomya albiceps* (Wiedemann) and reported similar landmark dispositions on the base of parastomal bar. The additional variations on landmarks found in the current study can be used as reference to conduct geometric morphometric analysis between *C. rufifacies* and *C. albiceps*, as both are biologically equivalent and difficult to distinguish the similarly looking 'hairy' larvae (Tantawi & Greenberg 1993; Wells & Sperling 1999; Adam Shahid et al. 2000; Grella et al. 2015). This will contribute to a more proper diagnosis in order to avoid misidentification between the two species especially when they are being utilized in forensic investigations.

Current study established 10 landmarks for geometric morphometric analysis compared to 8 landmarks used by Nuñez-Rodríguez and Liria (2017) with additional points on upper margin of ventral cornua (landmark 7), ventral apodeme of mouth hook (landmark 9) and basal hook (landmark 10). The union between hypostomal sclerite and the mouth hook, or landmark 5 in Nuñez-Rodríguez and Liria (2017), was not chosen in this study because the image obtained from stereomicroscope was obscure. For future study, we recommend acquiring clearer and better quality images by using compound microscope with adequate source of light.

It is also important to note that the selection of these landmarks demands further investigation to address the coplanarity issue because the three-dimensional shape of cephalopharyngeal skeleton could have been distorted when projected as two-dimensional image (Webster & David Sheets 2010; Zelditch et al. 2012). Furthermore, the conjoining pharyngeal sclerite and hook part could be exposed to movement during cleaning process (Rabbani & Zuha 2017) and subsequently affecting the landmark plots. We minimized these effects by mounting the cephalopharyngeal skeleton in Berlese fluid down to the depth nearest to the slides and use similar focusing level to obtain the image. Further confirmation of landmarks by using more detailed description of the cephalopharyngeal skeleton based on ultramicroscopic analysis could assist with the limitations in viewing the structures based on stereo and compound microscopes.

CONCLUSION

Geometric morphometric analysis provides more practical and useful tools to profile and discriminate *C. megacephala* and *C. rufifacies* cephalopharyngeal skeleton shape. This technique could be used as supplementary taxonomic information to improve its admissibility in court as scientific evidence (Suzana & Zuha 2018) but further studies are required to include more species representations and increase sample size. We suggest future research to use controlled environments because factors such as temperatures and food source may influence larval development including the cephalopharyngeal skeleton.

ACKNOWLEDGEMENTS

The authors would like to thank the laboratory staffs from Forensic Science Program, Faculty of Health Sciences, UKM for providing the equipment and chemicals required. We also wish to thank Ms. Sharifah Shakilah Abdullah for her kind assistance during the research and Dr. Rus Dina Rus Din for reviewing the early draft of this manuscript. This research was funded by UKM Research Incentive Grant: GGP-2017-036.

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