# WING MORPHOMETRIC ANALYSIS OF BLOW FLIES FOR SPECIES IDENTIFICATION: INTRA- AND INTER-INDIVIDUAL VARIATIONS IN *CHRYSOMYA MEGACEPHALA* (FABRICIUS) AND *CHRYSOMYA RUFIFACIES* (MACQUART)

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**Abstract.** Species identification of blow flies is the initial step used in forensic investigation. Identification techniques usually are based on morphology and molecular analysis, the former requiring an experienced taxonomist while the latter laboratory expertise and considerable expense. Landmark-based geometric morphometric analysis is being widely used as an alternative technique in species identification as it is simple to perform at low-cost and requires only basic training. However, it is recommended using the same individual in digitizing the landmark coordinates to reduce measurement error, but the results from using many individuals to digitize the landmark coordinates have not been addressed in detail. Here, intra-individual and inter-individual variations in the digitization wings of *Chrysomya megacephala* and *Chrysomya rufifacies*, the two forensically important blow fly species, were assessed. Five individuals digitized 19 landmarks on the right wing of each species using the same photograph, performed in duplicate. Analysis of the results using Procrustes ANOVA demonstrated low (8.9-19.0%) measurement error of intra-individual variations but higher (23.1%) for that of inter-individual variations. Based on discriminant function analysis, *C. megacephala* wing shape was clearly differentiated from that of *C. rufifacies* with high reliability. A cross-validation test indicated high (93.3-100%) accuracy of recognition of *C. megacephala* intra-individual variations and 100% accuracy for inter-individual variations, while that of recognition of *C. rufifacies* was 100% for both intra- and inter-individual variations. Thus, although measurement error was obtained from both intra- and inter-individual variations, correct identification of *C. megacephala* and *C. rufifacies* was achieved.

**Keywords:** *Chrysomya megacephala*, *Chrysomya rufifacies*, blow fly, geometric morphometric, identification, measurement error

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## INTRODUCTION

Blow flies (Diptera: Calliphoridae), *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart), are two of the most medically and forensically important fly species, and they are found in many parts of the world, occupying a wide range of environment, from urban settings to natural forest areas (Moophayak *et al*, 2014). As their habitat has a strong connection between unhygienic environment and human setting, adult flies are able to function significantly as mechanical carriers of pathogens capable of causing disease in humans (Greenberg, 1971), while larvae are myiasis-producing agents in both humans and animals (Zumpt, 1965). It is noteworthy that larvae of both *Chrysomya* spp are found colonizing human corpse, thereby providing entomological evidence in forensic investigations, *eg*, in estimating the minimum time since death and in determining the manner and cause of death (Sukontason *et al*, 2007; Amendt *et al*, 2011). In Malaysia, larvae of *C. megacephala* and *C. rufifacies* are the dominant maggots inhabiting human cadaver (Lee *et al*, 2004).

For use in forensic investigation, species identification of blow fly specimens found in and/or associated with human corpse is the initial step (Smith, 1986). Generally, identification of fly at the species level is based primarily on traditional morphology and molecular analysis; each technique has its advantages and drawbacks. For instance, morphology-based identification requires an experienced taxonomist (Kurahashi and Bunchu, 2011; Moophayak *et al*, 2011). On the other hand, although molecular identification is the most reliable method, it requires laboratory expertise and relatively higher expenditure.

The morphometric approach has received increasing acceptance due to its simplicity and low cost, requiring only a basic training (Changbunjong *et al*, 2016; Kluiters *et al*, 2016). The morphometric approach is divided into traditional and geometric morphometrics, the former involving direct measurement of the specimen size, while the latter the analysis of variations in shape using photography and computer scanning of the specimen. Geometric morphometrics is more rapid and easier to perform than traditional morphometrics (von Cramon-Taubadel *et al*, 2007).

Landmark-based geometric morphometric analysis of wings is increasingly applied in species identification of many insect taxa, *eg*, discrimination of Anopheline (Jaramillo-O *et al*, 2015; Gómez and Correa, 2017) and *Aedes* mosquitoes (Sumruayphol *et al*, 2016), *Lutzomyia* sand flies, (Giordani *et al*, 2017), *Phlebotomus stantoni*, and *Sergentomyia hodgsoni* (phlebotomine sand flies) (Sumruayphol *et al*, 2017), *Stomoxys* biting flies (Changbunjong *et al*, 2016), *Lycocerus*, *Prothemus*, and *Themus* beetles (Su *et al*, 2015), and *Apis* honey bees (Rattanawannee *et al*, 2010), and also to detect cryptic species of *Bactrocera tau* flies (Dujardin and Kitthawee, 2013).

Although landmark-based geometric morphometrics is a useful tool for species identification, reliability of this method is limited by measurement error, which can occur at any step of the procedure, the most common source of error being the digitizing step (Fruciano, 2016). Either landmark digitizing by the same person or specimen preparation by the same procedure is recommended (Hall *et al*, 2014; Sontigun *et al*, 2017). As regards measurement error, it is plausible to suppose that measurement error due to intra-individual variation (by the same

Table 1
Geographical locations and coordinates, and number of blow flies collected.

| Species | Province (location) | GPS reference | | Total no. of specimens[a] |
|---|---|---|---|---|
| | | Latitude | Longitude | |
| *Chrysomya megacephala* | Lampang (Doi Khun Tan) | 18°23′34.837″N | 99°12′54.186″E | 30 |
| *C. rufifacies* | Songkhla (Prince of Songkla University) | 7°0′32.60″N | 100°30′20.65″E | 30 |

[a]Both males and females.

individual) and/or inter-individual variation (among different individual) could constitute a hindrance to wing morphometric analysis. Consequently, this study assesses intra- and inter-individual variations in wing morphometric analysis of *C. megacephala* and *C. rufifacies*.

## MATERIALS AND METHODS

### Fly specimens

Adult *C. megacephala* and *C. rufifacies* were collected from Lampang and Songkhla Provinces, Thailand during 2015-2016 (Table 1). A total of 60 specimens were obtained using a sweep net and one-day-old beef offal (300 g) as bait. Males and females were combined and the specimens were euthanized, stored in 85% ethanol and identified from external morphology using the taxonomic key of Kurahashi and Bunchu (2011).

### Slide preparation

The right wing was removed from each fly and placed on a drop of Permount™ (Fisher Scientific, Waltham, MA) mounting medium on a microscope slide. One drop of xylene was placed to decrease the thickness of the mounting medium. Then, a cover slip was placed on top of the wing specimen and the slide was allowed to dry for a week at room temperature.

### Image processing and data acquisition

Digital images of each wing were taken using an AxioCam ICc1 camera (Zeiss, Jena, Germany) connected to a stereomicroscope (Olympus SZ61, Tokyo, Japan), 1.5 x magnification. TPS file of the images was constructed using the tpsUtil software version 1.64 (Rohlf, 2013) to minimize the possible bias in digitizing landmark locations. In order to capture the wing shape of *C. megacephala* and *C. rufifacies*, 19 landmarks were digitized in each wing digital image according to Hall *et al* (2014), avoiding landmarks on the proximal part of the wing (Fig 1). Then the set of the 19 landmarks was positioned using tpsDig2 software version 2.20 (Rohlf, 2015).

In order to assess the digitizing error, analyzed data were taken from the same database (same specimen, same photograph). Five individuals recorded the 19 landmarks on each wing. Repeatability tests on digitization were performed by capturing the images of 60 wings twice.

### Statistical analysis

The TPS file containing raw coordinates of landmarks for intra-individual and five inter-individual variations were subjected to analysis using a MorphoJ software version 1.06 (Klingenberg, 2011) and aligned using a Procrustes Fit function to remove differences in scale,

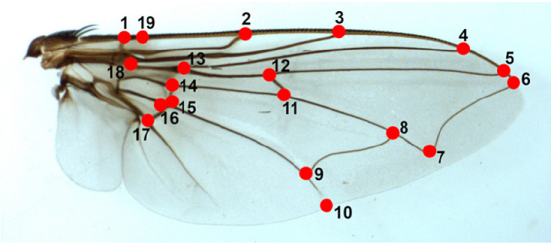


Fig 1–Right wing of *Chrysomya megacephala* showing positions of the 19 landmarks selected to describe the shape, based on Hall *et al* (2014).

position and orientation from the raw coordinates. Procrustes ANOVA in MorphoJ was applied to assess the intra- and inter-individual variations. The mean square (MS) values obtained from Procrustes ANOVA performed on intra-individual or five inter-individual variations were employed to quantify the intra- and the inter-individual measurement errors. Percent intra-individual measurement error (%ME) was calculated using the following formulas (Yezerinac *et al*, 1992):

$$\%ME = [S^2_{within}/(S^2_{within} + S^2_{among})] \times 100$$

$$S^2_{within} = MS_{within}$$

$$S^2_{among} = (MS_{among} - MS_{within})/m$$

where $S^2_{within}$ is the within-specimen component of variance, $S^2_{among}$ the among-specimen component of variance, $MS_{within}$ the mean square within the specimen, $MS_{among}$ the mean square among specimens, and m the number of repeated measurements.

Percent inter-individual measurement error (%ME) was calculated using the following formulas (Blackwell *et al*, 2006; Muñoz- Muñoz and Perpiñán, 2010):

$$\%ME = [S^2_{obs}/(S^2_{within} + S^2_{sp} + S^2_{obs})] \times 100$$

$$S^2_{sp} = (MS_{sp} - MS_{obs})/bm$$

$$S^2_{obs} = (MS_{obs} - MS_{rep})/m$$

$$S^2_{within} = MS_{rep}$$

where $S^2_{within}$ is the within-specimen component of variance, $S^2_{sp}$ the among-specimen component of variance, $S^2_{obs}$ the among-observer component of variance, $MS_{sp}$, $MS_{obs}$, and $MS_{rep}$ the mean square of the specimen, observer and replication component, respectively, and b the number of observers and m represents the number of repeated measurements.

In order to determine the difference in wing shape between *C. megacephala* and *C. ruffifacies* for both intra- and five inter-individual variations, the Procrustes coordinates obtained from the landmark data after Procrustes superimposition were averaged and assessed using a discriminant function analysis (DFA) and a cross-validation test in MorphoJ (Klingenberg, 2011). Statistical significance of the Mahalanobis distances was tested using a permutation test with 10,000 rounds. A significance level ($p$-value) of ≤0.05 is used for determining significant difference between groups.

## RESULTS

The Procrustes ANOVA of shape variation obtained from the intra-individual variation analysis showed the MS values for the among-specimen variation are highly significant ($p$ <0.0001) and much higher than the repeated landmark data, indicating that the digitizing error was negligible relative to the among-specimen variations (Table 2). The MS values revealed the smallest and the largest level of among-specimen variation was 9.49 and 21.41 times greater, respectively than the digitizing error. As for the inter-individual variation, there are significant differences between specimens ($p$ <0.0001) and between individuals ($p$ <0.0001), but not in the digitizing error. The MS value of among-specimen variation was 12.94

Table 2
Procrustes ANOVA for shape analysis of right wing of *Chrysomya megacephala* and *C. rufifacies* within (intra-) individual and between (inter-) individuals.

| Individual | Effect[a] | SS | MS | df | F | *p*-value |
|---|---|---|---|---|---|---|
| Intra-individual no. 1 | Individual (specimen) | 0.06454489 | 0.0000321759 | 2006 | 19.01 | <0.0001 |
| | Error 1 (digitizing error) | 0.00345241 | 0.0000016924 | 2040 | | |
| Intra-individual no. 2 | Individual (specimen) | 0.06604384 | 0.0000329232 | 2006 | 9.49 | <0.0001 |
| | Error 1 (digitizing error) | 0.00707675 | 0.0000034690 | 2040 | | |
| Intra-individual no. 3 | Individual (specimen) | 0.06659897 | 0.0000331999 | 2006 | 16.47 | <0.0001 |
| | Error 1 (digitizing error) | 0.00411169 | 0.0000020155 | 2040 | | |
| Intra-individual no. 4 | Individual (specimen) | 0.07117608 | 0.0000354816 | 2006 | 17.69 | <0.0001 |
| | Error 1 (digitizing error) | 0.00409227 | 0.0000020060 | 2040 | | |
| Intra-individual no. 5 | Individual (specimen) | 0.06356985 | 0.0000316899 | 2006 | 21.41 | <0.0001 |
| | Error 1 (digitizing error) | 0.00301934 | 0.0000014801 | 2040 | | |
| Inter-individual | Individual (specimen) | 0.31480562 | 0.0001569320 | 2006 | 12.94 | <0.0001 |
| | Error 1 (Observer error) | 0.09899588 | 0.0000121318 | 8160 | 5.69 | <0.0001 |
| | Residual (digitizing error) | 0.02174897 | 0.0000021323 | 10200 | | |

[a]For each dataset, the sum of squares (SS), mean square (MS), degree of freedom (df), F statistics and *p*-value are shown.

times greater than individual error, and the latter was 5.69 times greater than the digitizing error. The intra- and inter-individual percent measurement error (%ME) calculated using the MS values obtained from Procrustes ANOVA (Table 2) was 8.9-19.1 and 23.1, respectively (Table 3).

The difference in wing shape between *C. megacephala* and *C. rufifacies* using DFA is highly significant ($p$ <0.0001) based on Mahalanobis distances for both intra- and inter-individual variations (Table 4). The correct classification of *C. megacephala* was 93.3-100% and 100% for intra- and inter-individual variation, respectively, whereas that of *C. rufifacies* was 100% for both intra- and inter-individual variations (Table 4). Histogram of the scores for the cross-validation analysis did not overlap (Fig 2). After superimposition of the mean landmark configurations, the wireframe graph of the wing shape conformations of *C. megacephala* and *C. rufifacies* assessed by inter-individual analysis exhibited distinct difference (Fig 3).

Table 3
Percent measurement error of intra- and inter-individual variations in analyzing right wing of *Chrysomya megacephala* and *C. rufifacies*.

| Individual | Measurement error (%) |
|---|---|
| Intra-individual no. 1 | 10.0 |
| Intra-individual no. 2 | 19.1 |
| Intra-individual no. 3 | 11.5 |
| Intra-individual no. 4 | 10.7 |
| Intra-individual no. 5 | 8.9 |
| Inter-individual | 23.1 |

## DISCUSSION

Wing morphometry approach has been increasingly employed in insects to determine phenotypic variations at the inter-specific level (Dellicour *et al*, 2017). In flies, the landmark-based geometric morphometric method has been applied for taxonomic purposes (Pieterse *et al*, 2017). However, in this technique, measurement error might occur while digitizing the landmark coordinates of the wing in both intra- and inter-individual analysis. The present work assessed the intra-individual and inter-individual variations in this context.

Table 4
Percent specimens in *Chrysomya megacephala* and *C. rufifacies* correctly identified from cross-validation analysis using permutation test with 10,000 rounds in MorphoJ.

| Individual | Mahalanobis distance | $p$-value | Percent correctly classified ($n$ = 30) | |
|---|---|---|---|---|
| | | | *C. megacephala* | *C. rufifacies* |
| Intra-individual no. 1 | 12.7272 | <0.0001 | 97 | 100 |
| Intra-individual no. 2 | 13.1349 | <0.0001 | 100 | 100 |
| Intra-individual no. 3 | 12.2440 | <0.0001 | 100 | 100 |
| Intra-individual no. 4 | 11.2827 | <0.0001 | 93 | 100 |
| Intra-individual no. 5 | 11.5465 | <0.0001 | 100 | 100 |
| Inter-individual | 13.1105 | <0.0001 | 100 | 100 |

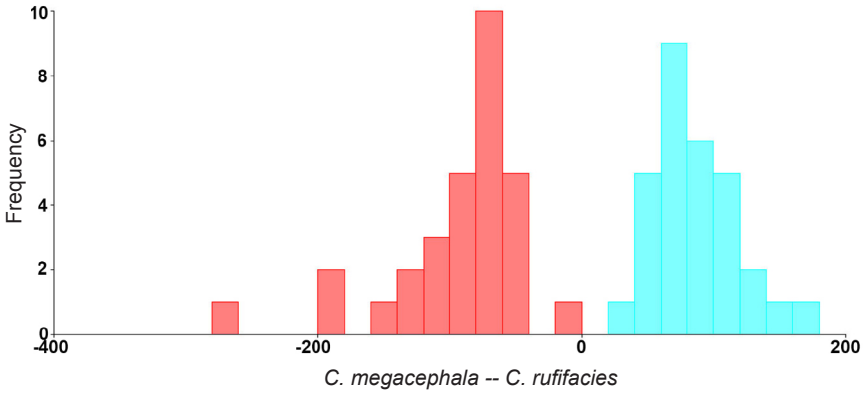


Fig 2–Histogram from cross-validation analysis of *Chrysomya megacephala* and *C. rufifacies*. Number on x-axis denoted cross-validation scores.

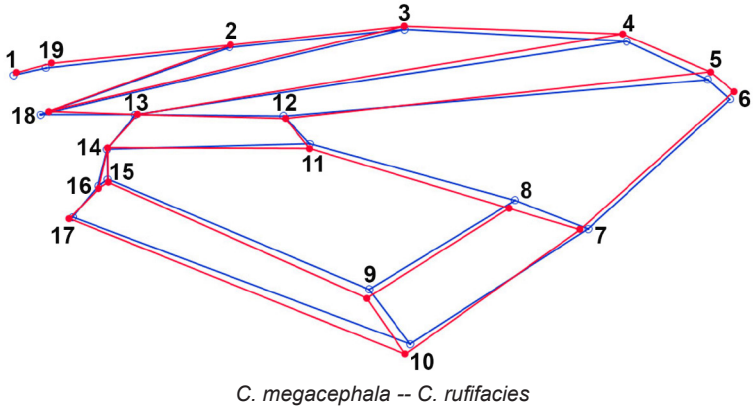


Fig 3–Wireframe graph of wing showing mean shape difference between *Chrysomya megacephala* and *C. rufifacies* after discriminant function analysis (DFA).

The very small differences in landmark placement (digitizing error) for intra-individual variation compared to the among-specimen variation as assessed using Procrustes ANOVA analysis indicates the landmarks used in the study can be located with precision. For the inter-individual variation, although a statistical significance of individual error was found, the MS of the individual error was small compared to the among-specimen variation. In this regards, shape variation was influenced by biological variation among individual specimens, which was larger than the measurement error due to the use of an individual observer and digitization.

The higher measurement error in the inter-individual variation than in the intra-individual variation indicated that individual error was greater than digitizing error. This phenomenon is in line with investigations in other animal taxa, *viz*. moths (Arctiidae, Gometridae and Noctuidae) (Goodenough *et al*, 2012), tsetse flies (*Glossina fuscipes* and *Glossina palpalis palpalis*) (Dujardin *et al*, 2010), cyprinid fish (*Alburnus, Carassius gibelio* and *Rutilus rutilus*) (Fruciano, 2016), and the higher taxon of Gorilla (Tocheri *et al*, 2011). It is worth noting that in these studies analysis of datasets obtained from different measurers was avoided.

Although the result of this study showed both intra- and inter-individual measurement variations in wing morpho-

metric analysis, interestingly, these variations did not affect species identification of *C. megacephala* and *C. rufifacies*. This is in line with the report of Sontigun *et al* (2017) using the same 19 landmarks in discriminating these two fly species from the ten other blow fly species; indeed, the percent correct classification of these two species showed similar results in intra- and inter-individual variations, indicating in these two species, data taken from multiple individuals can be used for species discrimination between them. However, more individuals, samples and/or species of flies will be needed to allow drawing of stronger conclusions. On the other hand, wing morphometric analysis of closely related species, cryptic species and/or species complex should be carried out with great care, avoiding the use of different individuals. For instance, Dujardin *et al* (2010) reported errors by individuals in the classification of two closely related species increase when multiple observers digitized the specimens. Nonetheless, use of data taken from multiple individuals may be necessary when sample sizes are large and/or individual researchers are constrained by limited budget/time. In addition, it is worth noting that errors in the identification of a sibling or a closely related species are also probably influenced by the method and the instrument used.

Besides personal errors caused by the observer (inter-individual) and digitization (intra-individual), methodological error (*eg*, mounting technique of specimens and preservation method), and instrument error (*eg*, photographing condition, data-acquisition device and measuring device) have significant effects on geometric morphometric studies (Fruciano, 2016). For example, a wing shape analysis of blow fly *C. bezziana* exhibits inter-method error from different preparations among wings on flies and those flattened on slides (Hall *et al*, 2014). In addition, assessment of four morphometric techniques, namely, traditional caliper-based method, truss network method, geometric method on the body, and geometric method on the scales, revealed that all morphometric methods are influenced by the measurer and that variations in the method employed also affected species identification (Takács *et al*, 2016). Examples of instrument error have been reported by Collins and Gazley (2017) in their studies of New Zealand Mactridae (bivalve shells) and who suggested that avoidance of mixing data sets from different cameras, lenses and photographic setups, and of placing specimens near the edges of photographs should be observed to reduce measurement errors.

In conclusion, in spite of variations in measurement between intra- and inter-individual wing morphometric analyses, correct identification of *C. megacephala* and *C. rufifacies* was achieved based on cross-validation analysis. This information should be useful in wing morphometric analysis of flies in cases where numerous specimens are collected from the field and a number of measurers are required to reduce the work load of an individual investigator.

## ACKNOWLEDGEMENTS

The work was supported by the Diamond Research Grant, Faculty of Medicine, Chiang Mai University. The authors are grateful to Chiang Mai University for defraying publication cost.

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