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Wing Morphometrics of Forensic Important Fly Species in Kelantan, Malaysia

Helmi HADI

Forensic Science Program, School of Health Sciences, University Sains Malaysia, 16150 Kubang Kerian, Kelantan, MALAYSIA
e-mail: helmi_mhp@usm.my
ORCID ID: 0000-0002-3225-8327

ABSTRACT

This research discusses the possibility of determining the species and sex of necrophagous flies by examining their wing morphology. Fresh beef meat (500 g) was placed in both rural and urban areas of Kelantan, Malaysia, and third instar maggots were collected for rearing from the third to the seventh day. Adult flies were killed with chloroform and their species and sexual status were determined using standard procedures. After detaching their wings, the morphology was documented using a Leica MC 170HD digital camera attached to a Leica stereo microscope. Thirteen wing landmarks were chosen for geometric morphometric analysis. Principal Component Analysis (PCA), canonical variate analysis (CVA), centroid size variation, and the unweighted pair-group method with arithmetic averages (UPGMA) were used to analyse the wing morphology of *Chyrsomya megacephala* (Fabricius, 1794), *Chrysomya rufifacies* (Macquart, 1843), and *Sarcophaga ruficornis* (Fabricius, 1794) The results indicate that sex and fly species can be distinguished based on wing morphology, and that CVA is more effective at species differentiation than sex grouping using Malahanobis distances, with a P value of 0.0001. When comparing different species and sexes of the fly, CVA demonstrates distinct clustering. Identification of necrophagous fly wings is a useful alternative tool for fly classification.

Keywords: Principal Component Analysis (PCA), Canonical variate analysis (CVA), *Chyrsomya megacephala , Chrysomya rufifacies, Sarcophaga ruficornis,* necrophagous fly, species identification

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INTRODUCTION

Necrophagous insects are particularly useful during forensic investigations as they help in estimating the post-mortem interval (PMI) in a scientifically acceptable manner whenever decomposing dead bodies are found at the crime scenes. These flies usually populate the dead body within hours of the body being exposed to the environment, provided the ambience is conducive for the fly to oviposit. Several types of research have been conducted on the necrophagous fly species in Malaysia (Abidin, 2013; Abu Bakar and Zuha, 2016; Chin et al, 2007; Kavitha et al, 2013; Mahat et al, 2009; Mahat et al, 2014; Morry, 2007; Pritam and Jayaprakash, 2009; Ting, 2005). Reports have been published summarizing the necrophagous fly species populating cadavers in different periods in Malaysia such as between 1972-2002 (Lee et al, 2004) and 2005 to 2010 (Kavitha et al, 2013) and in Thailand between 2002 to 2006 (Sukontason et al, 2007).

Morphometrics is quantitatively researching the relationship between biological shape, variation and covariation of shape and other abiotic or biotic factors (Webster and Sheets, 2010). Landmark based Geometric Morphometric (GMM) analysis has been conducted on butterfly wings (Roggero and Entreves, 2005; Breuker et al, 2010), various non-necrophagous fly wings (Klingenberg et al, 1998; Haas and Tolley, 1998; Hall et al, 2014; Pepinelli et al, 2013; Sadeghi and Kiany, 2012), Psychodidae (De la Riva et al, 2001), cricket wings (Klingenberg et al, 2010), culicid mosquito (Sanchez et al, 2017) and necrophagous fly wings (Vásquez and Liria, 2012; Macedo, 2016; Nuñez-Rodríguez and Liria, 2017; Sontigun et al, 2017).

There are various GMM methods for explaining the research data. The use of centroid size (CS) measurement for measuring allometry is useful when measuring the overall GMM landmark configuration provided that all specimens have the same landmark coordinates (Webster and Sheets, 2010). Principal Component Analysis is a useful tool for exploring the shape differences present in a landmarked sample (Webster and Sheets, 2010). The first principal component (PC) shows the most variation of the data followed by the second PC which is orthogonal to the first direction to showcase the most variation (Webster and Sheets, 2010). Canonical variates analysis (CVA) gives a graph output with specimens ordered along the created output. The differences between PCA and CVA are that CVA assumes that the specimens are grouped before the analysis is carried out and tests how well the data or shape in the pre-defined groups support the findings. The CVA tests assume that the data is multivariate and normally distributed and the groups share a common structure (Webster and Sheets, 2010).

Morphometric studies on categorising necrophagous fly species wings have been conducted in Venezuela (Vásquez and Liria, 2012; Nuñez-Rodríguez and Liria, 2017), Brazil (Macedo, 2016) and Thailand (Sontigun et al, 2017). Table 1 summarises the previous research on necrophagous fly species. There are various methods for recording insect wings for morphometric analysis. Wing harvesting could be destructive or non-destructive. Destructive techniques include removing the wings and flattening the wings to view under microscopes (Johnson et al, 2013; Bubliy et al, 2008; Demayo et al, 2011).

Table 1. Previous GMM research on necrophagous fly wing morphology of forensic interest.

Author	Type of species and amount studied (no. of female/male if indicated)	Amount of fly samples landmarked	No of geometric morphometric landmark	Type of Geometric morphometric analysis
Vásquez and Liria, 2012	*C. megacephala* (57) *C. albiceps* (111)	168	8	Procrustes superimposition and PCA
Macedo, 2016	*C. albiceps* (55) *C. megacephala* (42) *H. segmentaria* (42)	139	13	PCA, DA, CVA
Nuñez-Rodríguez and Liria, 2017	*L. cuprina* (21f/19m) *C. megacephala* (21f/19m) *C. albiceps* (35f/35m)	150	8	Procrustes superimposition, CS variation, CVA, SShD,
Sontigun et al, 2017	*C. megacephala* (29f/24m) *C. chani* (23f/17m) *C. pinguis* (10f/29m) *C. rufifacies* (22f/25m) *C. villeneuvi* (16f/23m) *C. nigripes* (15f/17m) *L. cuprina* (14f/15m) *L. papuensis* (23f/9m) *L. porphyrina* (7f/11m) *L. sinensis* (4f/4m) *H. ligurriens* (18f/14m) *H. pulchra* (3f/0m)	372	19	Procrustes superimposition, CVA, CS, SShD, DA with cross validation, UPGMA
Grzywacz, Ogiela and Tofilski, 2017	*A. nebulosa* (0f/14m) *E. cyanicolor* (0f/40m) *G. maculata* (0f/17m) *H. impuncta* (0f/35m) *M. levida* (0f/163m) *M. urbana* (0f/14m) *H. dentipes* (0f/318m) *M. domestica* (0f/46m) *N. cornicina* (0f/30m) *P. pallida* (0f/45m) *P.lardarius* (0f/8m) *S. calcitrans* (0f/32m) *T. simplex* (0f/28m)	790	15	MANOVA, CVA
Szpila, Żmuda, Akbarzadeh, & Tofilski, 2019	*C. rohdendorfi* (3f/7m *C. subalpina (3f/7m)* *C. vicina 85f/130m)* *C. vomitoria (38f/38m)* *C. albiceps (38f/24m)* *C. megacephala (49f/46m)* *C. mortuorum* (6f/27m) *L. ampullacea* (12f/19m) *L. Caesar* (40f/88m) *L. illustris* (8f/9m) *L. sericata* (72f/98m) *L. silvarum* (16f/27m) *P. Regina* (21f/9m) *P. terraenovae* (32f/12m)	968	15	PCA, MANCOVA, CVA, UPGMA
Jos, Jos, & Martínez-s, 2020	*C. vicina* (50f/50m) *C. vomitoria* (50f/50m) *C. albiceps* (50f/50m) *C. megacephala* (50f/50m) *L. caesar* (50f/50m) *L. sericata* (50f/50m)	600	17	PCA, Procustes ANOVA,
López-garcía, Angell and Martín-vega, 2020	*L. varipes* (20f/24m) *P. flavipes* (1f/5m) *P. vulgaris* (2f/6m) *P. casei* (24f/14m) *P. megastigmata* (12f/12m) *P. nigrimana* (26f/39m) *P. latipes* (5f/12m) *P. litigate* (5f/5m) *S. nigriceps* (7f/12m) *C. furcate* (1f/2m) *T. cynophile* (0f/5m)	239	14	PCA, CVA, DA, UPGMA

PCA Principal component analysis, DA Discriminant function analysis, CVA Canonical variates analysis, SShD Sexual shape dimorphism, CS Centroid size, UPGMA, Unweighted pair group method with arithmetic averages.

Non-destructive methods include orientating the insects so that the photographed wings are perpendicular compared to the microscope (Hall et al, 2014). Other non-destructive method includes the fabrication of a wing-capturing device (Perrard et al, 2012). A research on the accuracy of the different registering modalities (pinned,

scanned and detached wings) has been conducted on dragonfly wings (Johnson et al, 2013). It is crucial to standardise the wing harvesting and capturing procedure as differences will introduce errors (Johnson et al, 2013).

MATERIALS AND METHODS

Sample collection and preparation

This study was conducted between 24 March - 24 May 2016 in the rural and urban areas of Kota Bharu district (6° 8N, 102° 15E). Two fresh beef samples each weighing 500 g from animals that have been slaughtered two to four hours earlier were placed in a rural area of Dewan Beta and within the compounds of Universiti Sains Malaysia Kelantan. A slotted plastic basket with few bricks were placed on top of the basket to avoid interference from carnivores. The slots permitted free entry and exit of adult flies. A plastic sheet was placed covering the top half of the basket to protect the meat from the rain. The above setup and data collection were repeated another two times one week apart at the same location with new beef meat. Daily observation was conducted twice, and maggots were collected from the second day to the eighth day. Maggots collected twice a day were reared in suitably labelled ventilated containers. Weather data was collected from the local weather station (less than 2 KM from the study site). The temperature for the entire research period was recorded every day and it ranged between 24°C to 35°C.

Maggots were reared in 14 transparent plastic containers containing dry soil and a piece of decomposing meat. The top of the container was covered with gauze and secured with rubber bands to allow for ventilation while preventing the flies from escaping. Once the adult flies emerge from the pupae, a chloroform-soaked cotton ball was added to the plastic container and the container is closed tight with a wrapper for the chloroform to act on the flies. Species and the sex of the adult flies were noted based on available guidelines (Szpila, 2007) and the wings (left and right) were then removed carefully with tweezers for analysis. Damaged wings were excluded from the analysis. The removed wings were taped onto A4 papers with clear cellophane tape. The sample of flies studied during this research is shown in Table 2. The A4 papers were then scanned with an Epson L210 printer at 1200 dpi into jpeg images. Each wing image was cropped with Adobe® Photoshop® CS6 software for GMM analysis.

Geometric morphometric analysis in MorphoJ

Collected images were utilised to build a tps file by using TpsUtil V 1.74 software (Rohlf, 2005) before being landmarked in TpsDig2 V 2.30 software (Rohlf, 2005). Type I landmarks were placed on 13 points on the wing's venation. Type I landmarks are easily identified structures such as the intersection of two meeting points (Bookstein, 1997). Fig. 1. shows the GMM landmark location on the wing veins adopted for analysis as detailed in previous research (Rahman, 2015). Each wing image was duplicated further and was landmarked three times by the same individual to reduce the measurement error (Arnqvist and Martensson, 1998). The TPS file created was

uploaded into MorphoJ V 1.06d software (Klingenberg, 2011) and the raw landmarks were aligned and superimposed with the Procrustes Fit function based on the centroid size. The PCA and CVA were conducted on the species while Procrustes ANOVA was conducted on the centroid size and shape variation of the dataset. The CVA was conducted in order to identify the important features which can discriminate all the groups.

Table 2. Total number of specimens examined.

Fly species	Total no of specimens (male/female)
Chrysomya megacephala	136 (29/107)
Chrysomya rufifacies	97 (27/70)
Sarcophaga ruficornis	50 (0/50)

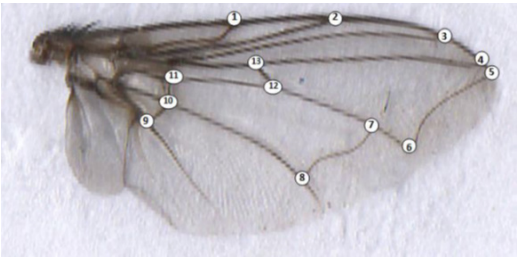

Figure 1. Wing of *Chrysomya megacephala* showing the 13 landmarks adopted for the morphometric analysis based on previous research (Rahman, 2015).

Size, shape, sex and phenetic relationship analysis

SPSS V 22.0 (IBM Corp. Armonk, NY) was utilised for the wing size, sex and sex difference based on fly species. The wing size was estimated with the Kruskal-Wallis H-test by utilising the mean centroid size, followed by the Mann-Whitney *U*-test (significance level of 0.05). The sex differences were tested with the Mann-Whitney *U*-test (significance level of 0.05). Boxplots of the centroid size by species and sex were produced to show the differences in the relative centroid size of the species.

In order to examine the phenetic relationship among the three fly species based on morphology, the unweighted pair-group method with arithmetic averages (UPGMA) was performed on the Procrustes Coordinates of the average dataset exported from MorphoJ in PAST V 3.17 (Hammer et al, 2001). The UPGMA dendrogram by Euclidian distance was constructed showing the relationship between the species analysed.

Sexual dimorphism

The sexual shape dimorphism (SShD) between each species for both males and females were tested by Mann-Whitney *U*-test in SPSS V 22.0 with a significance level of 0.05. The shape differences for females and males was analysed by Mahalanobis distances (with 10,000 permutations) was conducted in MorphoJ. Results are shown in Table 6.

RESULTS

Three species of necrophagous flies (n= 283) of *Chrysomya megacephala* (CM), *Chrysomya rufifacies* (CR) and *Sarcophaga ruficornis* (S) were collected from the reared maggots. Most of the species collected for the research were female flies (n= 227) compared to males (n= 56). No *Sarcophaga ruficornis males* were observed in the study (Table 2).

From the GMM PCA test, the Eigenvalues (Table 3) show that the top four Principal Components (PCs) account for 62.8% variation. The scatterplot graph of PC1 vs. PC2 of the wings are shown in Fig. 2 with the fly wing morphology at different ends (indicated by dotted box) of the x- and y-axes indicated by the selected dataset. The data for the male population (left polygon) and female population (right polygon) is shown for all fly species.

Table 3. Table showing Eigenvalues, percentage of variance and cumulative percentage variation of the Principal Components.

PC	Eigenvalues	% Variance	Cumulative %
1.	0.00027173	27.967	27.967
2.	0.00013921	14.328	42.294
3.	0.00011011	11.333	53.627
4.	0.00008891	9.151	62.778

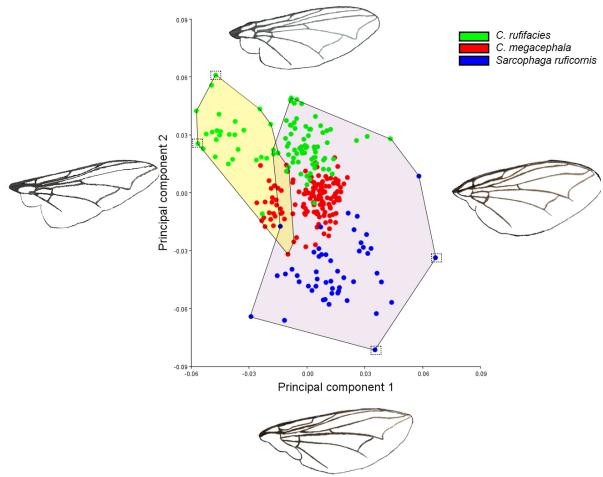

Figure 2. Scatterplot graph for PC1 and PC2 with the fly wing morphology which explains 42.3% of variance. The male fly population is shown in the left (yellow) polygon and female in the right (purple) polygon. The Rectangle box outline shows the fly morphology at extreme ends of the scatterplot.

Results for the size and shape are reported in Tables 4 and 5. Based on Table 4, the main effect of sex for size was almost 1% of the total sum of squares with a P-value of 0.0097. Differences in the fly species were larger and explained 60% with a significant P-value. The individual effect is lower compared to the fly species at 39%. For size variation, the fly species show the highest difference compared to the individual effect with sex having the smallest influence. Results for the shape (Table 5) mimic those for the size with the exception the sex being higher at 11% with a

significant P-value. The shape variation for the fly species and fly wings were 50% and 38% which was slightly lower compared to the centroid size variation.

The CVA indicates that the species and the sex are clearly distinct from each other (Fig. 3). Mahalanobis distances from Table 6 range from 3.60 (CM, (male vs. female)) to 16.05 (CR (male) vs S, (female)). All permutation tests indicate that the mean shapes vary among taxa with P <0.0001 in pairwise permutation tests (10000 permutation rounds) for Mahalanobis distances among groups. The Procrustes distances among groups range from 0.0269 (CM, (male vs. female)) to 0.1115 (CR (male) vs S, (female)). The scatter plot of CV shows a marked concentration at high CV1 scores for the Chrysomyinae subfamily. The shape of the wings differs in morphology on the CV1 axes and differs in size on the CV2 axes (Fig. 3).

Table 4. Centroid size variation.

Effect	Explained SS (%)	SS	MS	df	F	*p*
Sex	0.959	61769.922	61769.922	1	6.78	0.0097
Fly species	59.571	3835404.079	1917702.040	2	210.54	<0.0001
Fly wings (individual)	39.470	2541231.903	9108.358	279		
Total	100	6438405.904				

SS sum of squares, MS mean squares, df degree of freedom, F F statistics, p paramatic p-value.

Table 5. Shape variation.

Effect	Explained SS (%)	SS	MS	df	F	P	Pillai tr.	*p*
Sex	11.801	0.077	0.003	22	86.76	<.0001	0.76	<0.0001
Fly species	50.249	0.327	0.007	44	184.71	<.0001	1.68	<0.0001
Fly wings (individual)	37.950	0.247	0.000	6138				
Total	100	0.650						

SS sum of squares, MS mean squares, df degree of freedom, F F statistics, p paramatic p-value, Pillai tr. Pillai's trace.

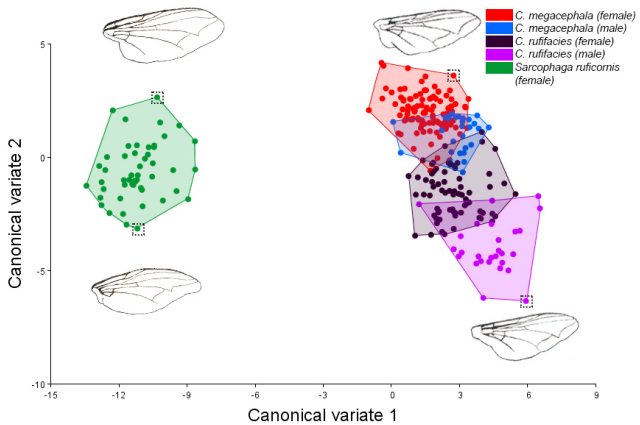

Figure 3. Canonical variate analysis of necrophagous fly wings. The fly wing shapes show the shapes for CV1 scores of -15 and +9 and CV2 scores of -10 and +5 (all other CV scores kept at value 0). The various wing morphology nearest to the dotted square box is displayed as well.

Table 6. Mahalanobis distances among groups for the specimens analysed with the sex (male/female). The p-values for all attributes were highlight significant (p<0.0001) with 10,000 permutation rounds in MorphoJ.

	CR (f)	CR (f)	S (f)	CM (m)
CR (f)	4.035			
S (f)	13.021	14.042		
CM (m)	3.602	4.848	14.410	
CR (f)	6.919	4.422	16.048	5.700

CR *C. rufifacies*, CM *C. megacephala*, S *Sarcophaga ruficornis*, f female, m male.

The centroid size among species were significantly different. Fig. 4. shows the boxplot with the centroid size of wings for the sex of each species. Only female S were available in this research. The Kruskal-Wallis Chi-square test is 114.026, *df* = 2, *P* = <0.001). There is no significant difference between males and females as the P value was > 0.05 (at 0.366). Post-hoc analysis was conducted between groups on the mean centroid size and the Mann-Whitney *U*-test between CM vs CR (7528.000), CR vs S (7153.000) and CM vs S (1275.000) were highly significant (P = <0.001). The UPGMA phenetic relationship between the samples revealed that the Chrysomyinae subfamily pairing close together compared to S (Fig. 5). The above results agree with the CV1 vs CV2 results in Fig. 3 wherein samples from the Chrysomyinae subfamily overlap compared to S.

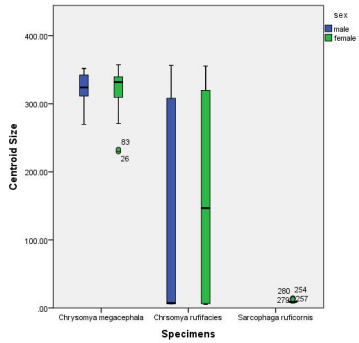

Figure 4. Boxplot showing the centroid size of wings for each fly specimen. Only female *Sarcophaga ruficornis* were collected in this research thus it could not be utilized for classifying between sexes. The mean centroid size Mann-Whitney U-test was highly significant at p <0.001.

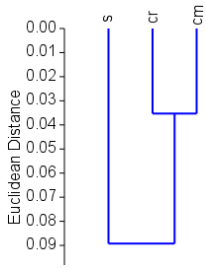

Figure 5. UPGMA dendrogram showing the fly wing morphology relationship construction based on Euclidean distances between specimens of *Chrysomya megacephala* (CM), *Chrysomya rufifacies* (CR) and *Sarcophaga ruficornis* (S).

DISCUSSION

Species-level identification of necrophagous species is fundamental for determining the time of death of an individual. CM and CR are the most common species in Kelantan followed by S. The amount and distribution of fly species obtained from this research are similar to the fly population available from other previous research (Lee et al, 2004; Mahat et al, 2009; Mahat et al, 2014; Morry, 2007) and case studies (Kavitha et al, 2013) in Malaysia. Based on previous findings (Kavitha et al, 2013), the majority of fly infestation on cadavers in Malaysia is single fly species (92.5%) while only 7.5% are double fly species. According to Kavitha et al. (2013) also, CR and CM were the most abundant species in Malaysia accounting for 82.8% of species recovered. These findings are replicated here and the most dominant species were CM and CR. No other species were recorded. *Sarcophaga ruficornis* only accounted for 17.7% of the total flies found which was slightly higher compared to the 10% documented previously (Kavitha et al, 2013). Despite the geographical proximity of Malaysia and Thailand, the variety of necrophagous fly species in Thailand (Sukontason et al, 2007; Zajac et al, 2016) is more compared to species found in Malaysia (Kavitha et al, 2013; Lee et al, 2004). Majority of the maggots obtained and reared were females. It is unknown why females were observed more than male flies. There were no male *Sarcophaga ruficornis* flies recovered from the reared specimens. A total of 227 females were collected compared to 56 males. From the reared specimens obtained, female flies were significantly more compared to male flies by almost three times. It is unsure why there is a small number of male flies for *C. megacephala* and *C. rufifacies* and why no males were obtained from the *Sarcophaga ruficornis* maggots reared. The lack or little male flies observed may be due to female maggots being oviposited more on the meat. There does not seem to be dominant fly sex trend based on previous findings (Nuñez-Rodríguez and Liria, 2017; Sontigun et al, 2017).

From Fig. 2 and Table 3, the first four PC cumulative variation value 62.8%. The cumulative variation values for PC 5 to PC 22 amount to 37.2%. The fly morphology difference for PC1 against PC2 is shown in Fig. 2 for the extreme ends of the PC axes. Figure 2 shows that the wing morphology of male flies tend to be narrower and longer particularly between the intersection of veins Costa and Media (landmark 5) and Intersection of crossvein media-cubitus and vein cubitus (landmark 8). The PCA results show that there is a distinct difference between the wing shape morphology of the three different fly species recorded. Differences also extend into the sex morphology. However, as there are no male S flies collected, it is unknown how the wing morphology of the wings would differ further.

The CVA (Fig. 3) show the grouping of males and females Chrysominae flies and the difference in the grouping of the S flies. The species variation is closer compared to the sex variation as the clustering of both CM and CR were adjacent. The Kruskal-Wallis test followed by the post-hoc Mann-Whitney *U*-test show significant differences when analysing the mean centroid size of each species. However, there is no significant difference when comparing the wing size intra-species. The results for the CM wings

obtained are similar compared to the results by Sontigun et al. (2017) in Thailand Some of the CM wings were collected from Phatthalung and Trang which is nearer to Kota Bharu. However, they noted that there is a significant difference in the wing size variation for CR (Sontigun et al, 2017). The CR fly population is only available in Chiang Mai which is further north compared to Trang and Phatthalung (Sontigun et al, 2017) which is nearer to Kota Bharu. It seems that the CR population is available sporadically and may not be present throughout Thailand. It also has to be noted that S is not available anywhere in Thailand. The *Sarcophaga* genus may be present in Thailand but was not observed in the research (Sontigun et al, 2017).

The use of fly wing morphology for species identification in a forensic investigation has not yet been explored by researchers and practitioners. Previous researches indicate that wing venation can help determine the species of a fly (Nuñez-Rodríguez and Liria, 2017; Sontigun et al, 2017). It is usually easier to recover the whole fly sample from a crime scene compared to just the wings which are quite transient. The wing morphology may be useful in additional information on species identity for cases where flies obtained from a crime scene are severely damaged. When a dead fly degrades over time, the body of the fly is susceptible to fungus in humid conditions and withers in dry conditions. In this situation, the wings are the only organ available to identify the species of the fly. The overlap of the species clusters in the canonical variates analysis (Fig. 3) indicates that it is harder to differentiate between sexes of the same species compared to species that are different. While differences do exist, there is an overlap between the groups that have to be considered.

Wing morphometric analysis on the sex and species of forensic important fly species in Malaysia could be conducted with the aid of geometric morphometrics. The shape differences are useful for class classifying the fly species which populates the cadaver. If any fly wings were to be found at the crime scene, then the fly wing morphology is useful in determining the type of species which populates the cadaver. However, it has to be noted that acquiring fly wings may be difficult compared to the whole fly as it is quite small and light enough to be dispersed by the wind.

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