

Wing morphometrics of medically and forensically important muscid flies (Diptera: Muscidae)

Kwankamol Limsopatham^a, Tunwadee Klong-klaew^b, Nuttanon Fufuang^a, Sangob Sanit^a, Kabkaew L. Sukontason^a, Kom Sukontason^a, Pradya Somboon^a, Narin Sontigun^{c,d,*}

^a Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

^b Department of Medical Technology, School of Allied Health Science, Walailak University, Nakhon Si Thammarat 80161, Thailand

^c Akkhraratchakumari Veterinary College, Walailak University, Nakhon Si Thammarat 80161, Thailand

^d Centre for One Health, Walailak University, Nakhon Si Thammarat 80161, Thailand

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ABSTRACT

Many muscid flies (Diptera: Muscidae) are well-known as medical, veterinary, and forensically significant insects, thus correct species identification is critically important before applying for fly control and determining a minimal postmortem interval (PMI_{min}) in forensic investigations. Limited in taxonomic keys and taxonomists, as well as scanty in advanced molecular laboratories lead to difficulty in identification of muscids. To date, a landmark-based geometric morphometric analysis of wings has proven to be a promising alternative technique for identifying many insect species. Herein, we assessed wing morphometric analysis for identification of six medically and forensically important muscids, namely *Musca domestica* Linnaeus, *Musca pattoni* Austen, *Musca ventrosa* Wiedemann, *Hydrotaea chalcogaster* (Wiedemann), *Hydrotaea spinigera* Stein, and *Dichaetomyia quadrata* (Wiedemann).

A total of 302 right wing images were digitized based on 15 homologous landmarks and wing shape variation among genera and species was analyzed using canonical variate analysis, whereas sexual shape dimorphism of *M. domestica*, *M. ventrosa*, and *D. quadrata* was analyzed using discriminant function analysis. The cross-validation revealed a relatively high percentage of correct classification in most species, ranging from 86.4% to 100%, except for *M. pattoni*, being 67.5%. Misidentifications were mainly due to cross-pairings of the genus *Musca*; *M. domestica* VS *M. pattoni* VS *M. ventrosa*. The accuracy of classification using cross-validation test demonstrated that wing shape can be used to evaluate muscid flies at the genus- and species-level, and separate sexes of the three species analyzed, with a high reliability. This study sheds light on genus, species, and sex discrimination of six muscid species that have been approached using wing morphometric analysis.

1. Introduction

Flies in the family Muscidae (Order Diptera) are medically and forensically important insects worldwide. Adult of muscid flies are not only a nuisance but serve as mechanical carriers of various pathogens to humans (Baldacchino et al., 2013; Bahrndorff et al., 2017; Khamesipour et al., 2018); while larvae are myiasis-producing in humans and animals, e.g., house fly, *Musca domestica* Linnaeus (Ferraz et al., 2010; Dehghani et al., 2012). Since larval behavior of muscids often decomposes organic matter of animal and plant origins, some species decompose human remains, thereby indicating forensic importance. Reports of stages in the life cycle (egg, larva, pupa, and/or adult) of muscids found associating

with the human corpses and/or death scenes (e.g., active to dry decomposition stages) increased in many regions of the world. Examples of these have been provided by *M. domestica* in China, Saudi Arabia; *Muscina stabulans* (Fallén) in Egypt; *Hydrotaea spinigera* Stein in Thailand, China, Malaysia; *Hydrotaea similis* Meade and *Hydrotaea dentipes* (Fabricius) in Sweden (Grzywacz et al., 2014; Wang et al., 2018; Tantawi et al., 2018; Al-Khalifa et al., 2020). In addition, *Synthesiomyia nudiseta* (van der Wulp) was often found in the indoor case scenario (commonly advanced to dry decomposition stages), as reported from Thailand, Malaysia, USA, Italy, Spain (Sukontason et al., 2007; Velásquez et al., 2013; Syamsa et al., 2015; Pinto et al., 2017; Sanford, 2017). Besides human cadavers, *Musca sorbens* (Wiedemann), *Musca pattoni*

* Corresponding author.

E-mail address: narin.so@mail.wu.ac.th (N. Sontigun).

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Table 1
List of Thai muscid fly specimens used in the wing morphometric assessment.

Species	Species code	Collection site	No. of specimens		
			Males	Females	Total
<i>Dichaetomyia quadrata</i>	DQ	Forest area	5	19	24
		Longan orchard	4	20	24
		Palm plantation	1	3	4
		Total	10	42	52
<i>Hydrotaea chalcogaster</i>	HC	Forest area	0	7	7
		Longan orchard	0	9	9
		Palm plantation	0	32	32
		Total	0	48	48
<i>Hydrotaea spinigera</i>	HS	Forest area	0	6	6
		Longan orchard	0	9	9
		Palm plantation	0	32	32
		Total	0	47	47
<i>Musca domestica</i>	MD	Forest area	1	13	14
		Longan orchard	9	24	33
		Palm plantation	0	12	12
		Total	10	49	59
<i>Musca pattoni</i>	MP	Forest area	0	21	21
		Longan orchard	0	19	19
<i>Musca ventrosa</i>	MV	Forest area	4	46	50
		Longan orchard	3	0	3
		Palm plantation	3	0	3
		Total	10	46	56

Austen, *Musca ventrosa* (Weidemann), *M. domestica*, *M. stabulans*, *Hydrotaea chalcogaster* (Wiedemann), *H. spinigera*, *S. nudiseta*, *Atherigona orientalis* (Schiner), and *Atherigona* spp. were collected on animal carcasses – the animal model used in forensic entomology investigations (Sukchit et al., 2015; Moophayak et al., 2017; El-Gawad et al., 2019). Valuable applications were based on the development patterns of flies to estimate the PMI_{min}; however, few studies of muscid (e.g., *M. domestica*, *H. spinigera*, *S. nudiseta*) development have been published so far (Kumara et al., 2009; Velásquez et al., 2013; Wang et al., 2018, 2021). Although muscid flies are of medical and forensic importance, they have received little attention in medico-legal researches due to the limitation of species identification and lack of relevant information regarding the development of immature stages in Thailand.

Correct fly species identification is a prerequisite step for any implementations, such as designing effective control programs and calculating the PMI_{min} accurately. Generally, the identification of muscids is mainly based on traditional morphological characteristics (Tumrasvin and Shinonaga, 1982; Nihei et al., 2009; Sawaby et al., 2018; Gregor et al., 2019) and advanced molecular approach (Renaud et al., 2012; Ren et al., 2018; Achint and Singh, 2021; Grzywacz et al., 2021). However, the identification based on morphological taxonomics is relatively difficult in practice for non-experts. For DNA identification, it is not only an expensive methodology, but requires advanced equipment, leading to problems in the absence of a molecular laboratory. Furthermore, reference DNA libraries for species identification still do not cover all muscid taxa, causing some unknown specimens cannot be assigned to known species.

Progress has been made to facilitate species identification of insects. Similar to morphology-based and DNA-based identification methods, a landmark-based geometric morphometric analysis of insect wings has proven to be a promising alternative approach for not only genus and species identification, but also geographic and sex discrimination of a species (de Camargo et al., 2015; Oliveira-Christe et al., 2020; Sauer et al., 2020). This technique allows separating species of diversified insects of medical, veterinary, and forensic importance, such as blow flies (Lyra et al., 2010; Sontigun et al., 2017), muscid flies (Changbunjong et al., 2016; Grzywacz et al., 2017; Nuñez et al., 2019), flesh flies (Sontigun et al., 2019), and mosquitoes (Wilke et al., 2016; Sauer et al., 2020), as it is fast, relatively low-cost, and trustworthy. To date, few wing morphometric analyses of muscid flies have been published, e.g., biting (Changbunjong et al., 2016) and non-biting flies (Grzywacz et al., 2017; Nuñez et al., 2019), with limited in those involving forensically-relevant species. To address this issue, we aim to assess a landmark-based geometric morphometric analysis of wings for species identification of medically and forensically important non-biting muscids of Thailand. Additionally, sexual dimorphism based on wing traits was analyzed.

2. Materials and methods

2.1. Specimen collection

A total of 302 muscid flies used in this study were collected from three different microhabitats, including a forest area (N18°46'01.08", E98°56'08.3"; 344 m altitude), a palm plantation (N18°45'27.841", E98°55'48.515"; 330 m altitude) and a longan orchard (N18°45'56.66", E98°55'40.13"; 374 m altitude) in a suburban area of Chiang Mai province, northern Thailand during 2013–2014 (Table 1) as methodology previously described by Klong-klaew et al. (2017). Specimens were pinned and identified to species level based on taxonomic keys of Tumrasvin and Shinonaga (1978, 1982) under a stereo microscope

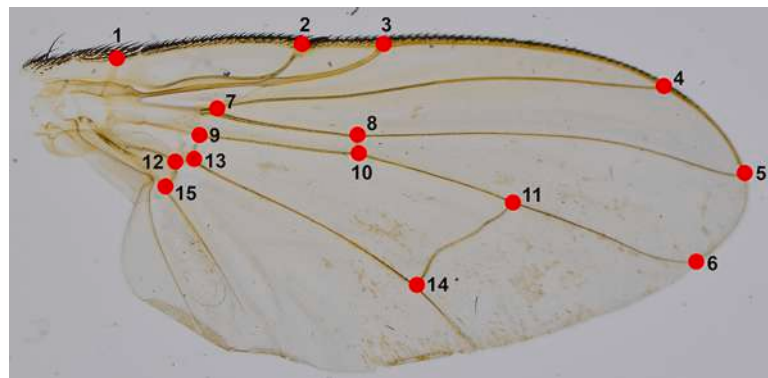


Fig. 1. The right wing of female *D. quadrata* plotted with 15 landmarks based on, as presented with red dots in number 1 to 15 of each landmark. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Olympus, Japan), including *Dichaetomyia quadrata* (Wiedemann), *Musca domestica* Linnaeus, *Musca pattoni* Austen, *Musca ventrosa* Wiedemann, *Hydrotaea chalcogaster* (Wiedemann), and *Hydrotaea spinigera* Stein (Table 1).

2.2. Slide preparation

The right wing of each fly was detached from the body with fine forceps, cut basicostae off from the wing using a scalpel blade, and mounted on a microscope slide according to the protocol by Sontigun et al. (2017).

2.3. Image processing and data acquisition

Each wing was photographed with a Nikon D5100 digital camera connected with Olympus SZ51 stereomicroscope at 1.5× magnification. All images of wings were kept in the same folder and then transformed as a tps file using TpsUtil software version 1.76 (<http://life.bio.sunysb.edu/morph/>) for further digitizing landmark locations. Fifteen landmarks as utilized by (Fig. 1) were placed on each wing using TpsDig2 software version 2.31 (<http://life.bio.sunysb.edu/morph/>) by the well-trained same person (NS). To eliminate measurement errors in the processing of wing images (Armqvist and Mårtensson, 1998), each wing was digitized twice by the same person (NS).

2.4. Geometric morphometric analysis

The tps file with raw coordinates of landmarks covering two replicates of digitization of individual specimens was imported into the MorphoJ software version 1.06 (Klingenberg, 2011). The raw coordinates of all specimens were initially superimposed using Procrustes Fit function to remove differences in scale, position, and orientation from the coordinates. After the Procrustes superimposition, the Procrustes coordinates and the centroid size (the square root of the sum of the squared distances between the center of configuration of the landmarks and each landmark) (Bookstein, 1991) were averaged for each specimen to eliminate any possible measurement error before further statistical analyses. All statistical tests were considered significant at the $P < 0.05$ level.

2.4.1. Allometry

To examine the relationship between wing size on wing shape variation (allometry) (Dujardin, 2008), the regression of Procrustes coordinates (dependent variable) on the centroid size (independent variable) was analyzed among species, pooled within species, and for individual species separately, using a permutation test with 10,000 rounds in MorphoJ software version 1.06 (Klingenberg, 2011). Allometric effects in sexual shape dimorphism (SShD) were measured by the regression of the Procrustes coordinates, pooled by sex, on the centroid size. As allometry can influence interspecific variation and sexual shape dimorphism (Gidaszewski et al., 2009; Sontigun et al., 2017), we used the residuals of the regression of shape on size for assessing the differences in shape without the size effect (allometry-free variables) in all subsequent analyses.

2.4.2. Shape variation

Canonical variate analysis (CVA) was conducted to assess wing shape differences among genus and species, while discriminant function analysis (DFA) was performed to determine sexual shape dimorphism (SShD) of three species, *D. quadrata*, *M. domestica*, and *M. ventrosa*. Differences in wing shape were analyzed based on Mahalanobis distances and tested with a permutation test (10,000 rounds) using MorphoJ software version 1.06 (Klingenberg, 2011). Additionally, the accuracy of classification was validated using a cross-validation test in DFA based on Mahalanobis distances and tested with a permutation test (10,000 rounds) using MorphoJ software version 1.06 (Klingenberg,

Table 2

Percentage of prediction indicating amount of size-related shape variation of wings in each muscid species and between sexes of each species performed by the regression of Procrustes coordinates on the centroid size using a permutation test with 10,000 rounds in MorphoJ.

Species	% predicted within species	P-value	% predicted between sexes	P-value
<i>D. quadrata</i>	1.18	0.5887	1.80	0.4921
<i>H. chalcogaster</i>	7.0	0.0004	–	–
<i>H. spinigera</i>	4.53	0.0523	–	–
<i>M. domestica</i>	0.78	0.8549	0.99	0.7793
<i>M. pattoni</i>	3.41	0.221	–	–
<i>M. ventrosa</i>	11.06	<0.0001	3.18	0.0954

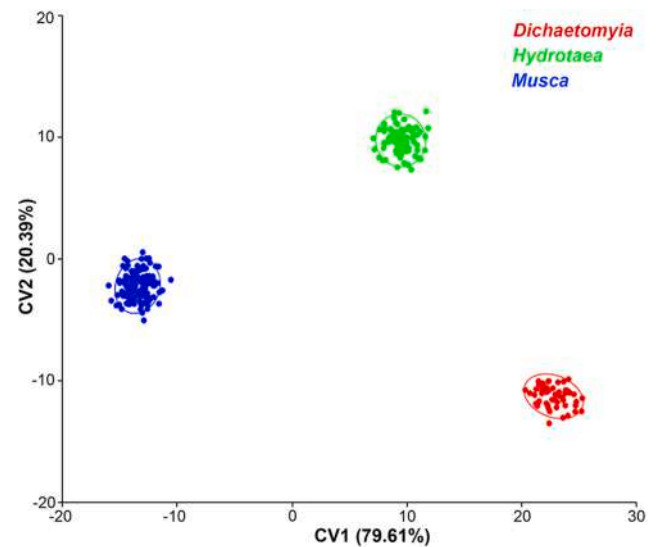


Fig. 2. Scatter plots showing wing shape variation of three muscid fly genera (red, green, and blue circles) along the first two canonical variates (CV1 and CV2) axes with 90% confidence ellipses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2011).

3. Results

3.1. Allometry

Regression of Procrustes coordinates on centroid size among species revealed highly significant differences (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$), allometry explained 1.60% of the total shape variation. Of the six analyzed species, allometry was detected only in *H. chalcogaster* and *M. ventrosa* (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$ and $P < 0.01$) (Table 2). Besides, the effect of size on shape variation was not found between sexes of the three analyzed species (permutation test with 10,000 rounds in MorphoJ: $P > 0.05$) (Table 2).

3.2. Wing shape variation

The results of morphometric analysis of the wing shape were visualized by CVA, of which allometry-free variables revealed wing shape differences among genus and species. The scatter plots demonstrated that individual specimens of each genus clustered into their respective groups and each genus clearly separated from each other (Fig. 2). Mahalanobis distances obtained from pairwise comparisons of all three genera revealed highly significant differences (permutation test with

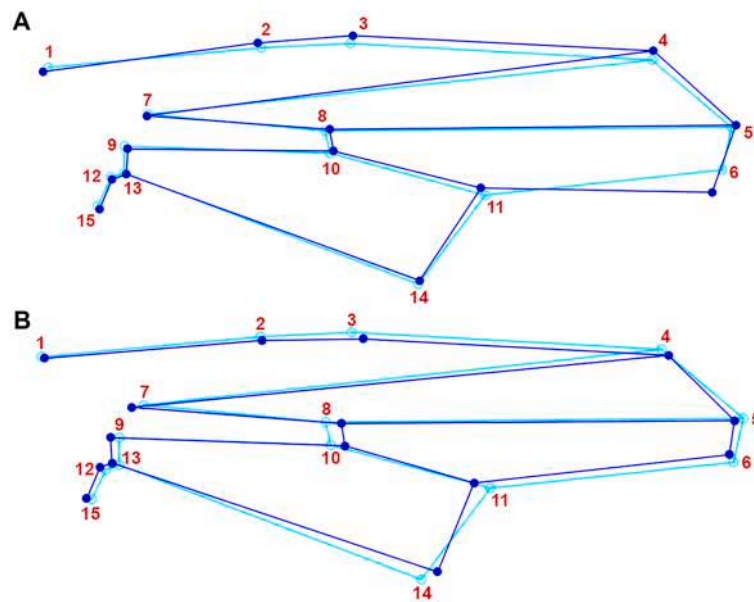


Fig. 3. Wireframe graphs displaying landmark displacements from the overall mean shape among genera along CV1 (A) and CV2 (B) axes in positive directions (increased 10 times). Light blue represents the mean shape of the samples and dark blue represents the landmark displacements of the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Percentages of correct classification obtained from pairwise comparisons of analyzed genera with a cross-validation test using a permutation test with 10,000 rounds in MorphoJ.

	Classified as			Total	Correct identifications (%)
	<i>Dichaetomyia</i>	<i>Hydrotaea</i>	<i>Musca</i>		
<i>Dichaetomyia</i>	52	–	–	52	100
<i>Hydrotaea</i>	–	95	–	95	100
<i>Musca</i>	–	–	155	155	100

Rows represent a given genus and columns represent a predicted genus.

10,000 rounds in MorphoJ: $P < 0.0001$), including 24.8748 (*Dichaetomyia* and *Hydrotaea*), 25.8659 (*Hydrotaea* and *Musca*), and 37.4141 (*Dichaetomyia* and *Musca*). Wireframe graphs in Fig. 3 illustrate shape changes along CV1 axis were clearly observed using landmarks 3, 4, 6,

and 11, whereas shape changes along CV2 axis were clearly found in most landmarks, except for landmarks 1, 2, and 15. The accuracy of classification estimated with cross-validation test was highest as 100% of correctly classified specimens in all genera (Table 3).

At the species level, the CVA revealed five canonical variates, of which the first two canonical variates (Fig. 4) demonstrated as high as 97.64% of the total shape variation (CV1 = 78.20%, CV2 = 19.44%). The scatter plot from CV1 and CV2 (Fig. 4) revealed that *D. quadrata* was clearly separated from the other five species, while the remaining species showed overlap among species, particularly between species within the same genus. Slight overlaps were observed between *H. chalcogaster* and *H. spinigera*, while *M. domestica*, *M. pattoni*, and *M. ventrosa* overlapped predominantly (Fig. 4). Mahalanobis distances obtained from pairwise comparisons of all six species had highly significant differences (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$) (Table 4), ranging from 3.8158 (*M. domestica* and *M. pattoni*) to 41.9403 (*D. quadrata* and *M. pattoni*). Wireframe graphs in Fig. 5 illustrate shape changes along CV1 axis obviously detected with landmarks 3, 4, 6, and

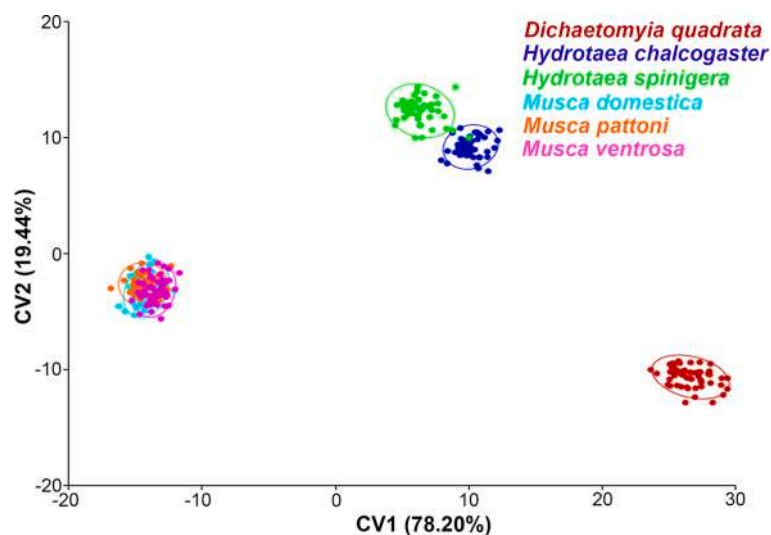


Fig. 4. Scatter plots showing wing shape variation of six muscid fly species along the first two canonical variates (CV1 and CV2) axes with 90% confidence ellipses.

Table 4

Mahalanobis distances (bold) and *P*-values (narrow) obtained from pairwise comparisons of analyzed species with CVA using a permutation test with 10,000 rounds in MorphoJ.

	DQ	HC	HS	MD	MP	MV
DQ	–	26.173	30.7482	41.6024	41.9403	42.0184
HC	<0.0001	–	8.6584	27.6612	27.8427	27.0255
HS	<0.0001	<0.0001	–	26.007	25.811	25.7653
MD	<0.0001	<0.0001	<0.0001	–	3.8158	4.5271
MP	<0.0001	<0.0001	<0.0001	<0.0001	–	5.1285
MV	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–

Abbreviations: DQ, *D. quadrata*; HC, *H. chalcogaster*; HS, *H. spinigera*; MD, *M. domestica*; MP, *M. pattoni*; and MV, *M. ventrosa*.

Table 6

Percentages of correct classification between sexes of three muscid species obtained from a cross-validation test using a permutation test with 10,000 rounds in MorphoJ.

Species	% correctly classified (no. of correctly classified/total no. of specimens)		
	Males	Females	<i>P</i> -value
<i>D. quadrata</i>	100 (10/10)	100 (42/42)	<0.0001
<i>M. domestica</i>	70 (7/10)	98 (48/49)	<0.0001
<i>M. ventrosa</i>	60 (6/10)	95.7 (44/46)	<0.0001

easily confused with *M. domestica*; and four specimens of *M. pattoni* were misidentified as *M. ventrosa*.

3.3. Sexual shape dimorphism

Wing shape between males and females analyzed by DFA showed highly statistical significance in all three species (permutation test with 10,000 rounds in MorphoJ: *P* < 0.0001) (Table 6). Visualized shape differences between males and females revealed that male wings were narrower than that of females (Fig. 6). The accuracy of classification after a cross-validation test for females ranged from 95.7% (*M. ventrosa*) to 100% (*D. quadrata*), whereas the accuracy of classification for males ranged from 60% (*M. ventrosa*) to 100% (*D. quadrata*) (Table 6).

4. Discussion

Wing shape has proven to be a valuable character as it was successfully used for genus and species discrimination in many insects (Grzywacz et al., 2017; Sontigun et al., 2019; Sauer et al., 2020). Therefore, it was hardly surprising that wing shape could be utilized to distinguish medically and forensically important non-biting muscids of Thailand at both the genus and the species levels. Analysis of wing shape variation showed that *D. quadrata* was clearly separated from *Hydrotaea* spp. and *Musca* spp. with 100% correct classification of individual genera. However, *Hydrotaea* spp. and *Musca* spp. displayed overlapping in the morphospace of canonical variates, especially *Musca* spp., leading to a lower percentage of correct assignment (67.5% to 86.4%). As misidentification occurred within *Hydrotaea* spp. and *Musca* spp., species identification merits performing in combination with other methods, such as taxonomic morphological keys or DNA analysis.

This study detected a significant sexual shape dimorphism in the three analyzed species (*D. quadrata*, *M. domestica*, and *M. ventrosa*) in having a high percentage of correct classification for females (95.7%–100%) and males (60%–100%). This finding suggested that wing morphologies are sex-specific and could be applied for sex discrimination. Since misidentifications were observed between wing shapes of males and females, it is recommended to apply wing morphometric analysis in conjunction with the characteristics of the eyes and/or genitalia for correct sex classification. In addition, our result demonstrated that males had considerably narrower wings than females (see Fig. 6), correlating with previous investigations, as female wings are usually larger or broader than males (Lyra et al., 2010; Virginio et al., 2015; Sontigun et al., 2019). Differences in SSHd might be correlated to flight performance, such as dispersal, migration, territoriality, courtship, and predator avoidance, as seen in other insects (Paquette et al., 2008; Devries et al., 2010). In this regard, more researches merit investigations whether the difference in SSHd is related to flight behavior of muscid flies.

As allometry can affect morphological variation among species, within a species, and between sexes of a species (Gidaszewski et al., 2009; de Camargo et al., 2015), the estimation of the allometric effects should be assessed before performing further shape analyses. In the present study, we eliminate the allometric effects from the data prior to shape analyses; therefore, differences in wing shapes between genus,

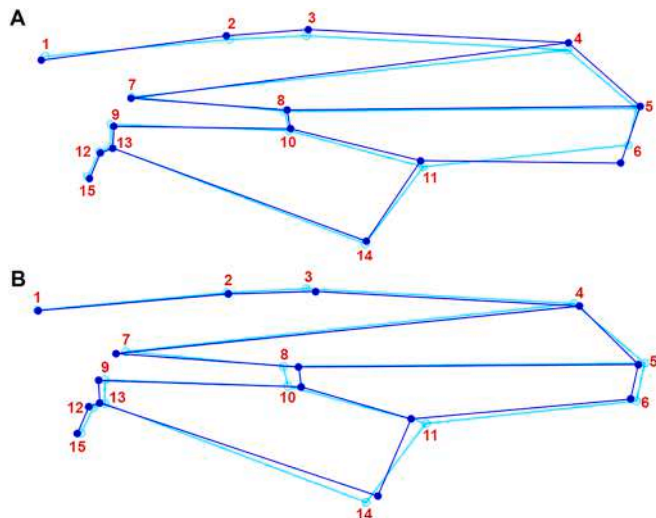


Fig. 5. Wireframe graphs showing landmark displacements from the overall mean shape among species along CV1 (A) and CV2 (B) axes in positive directions (increased 10 times). Light blue represents the mean shape of the samples and dark blue represents the landmark displacements of the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5

Identification error and percentage of correct classification obtained from pairwise comparisons of analyzed species with a cross-validation test using a permutation test with 10,000 rounds in MorphoJ.

	Classified as						Total	Correct identifications (%)
	DQ	HC	HS	MD	MP	MV		
DQ	52	–	–	–	–	–	52	100
HC	–	48	–	–	–	–	48	100
HS	–	1	46	–	–	–	47	97.9
MD	–	–	–	51	7	1	59	86.4
MP	–	–	–	9	27	4	40	67.5
MV	–	–	–	–	–	56	56	100

Rows represent a given species and columns represent a predicted species. Values in bold and narrow are correct and incorrect identification, respectively. Abbreviations: DQ, *D. quadrata*; HC, *H. chalcogaster*; HS, *H. spinigera*; MD, *M. domestica*; MP, *M. pattoni*; and MV, *M. ventrosa*.

11; whereas shape changes along CV2 axis were clearly observed using landmarks 8, 10, 11, and 14. The cross-validation test indicated a relatively high percentage of correct classification in most species (86.4% to 100%), except for *M. pattoni* (67.5%). With regard to misidentification (Table 5), one specimen of *H. spinigera* was misidentified as *H. chalcogaster*; seven specimens of *M. domestica* were relatively easily confused with *M. pattoni*; one specimen of *M. domestica* was misidentified as *M. ventrosa*; nine specimens of *M. pattoni* were relatively

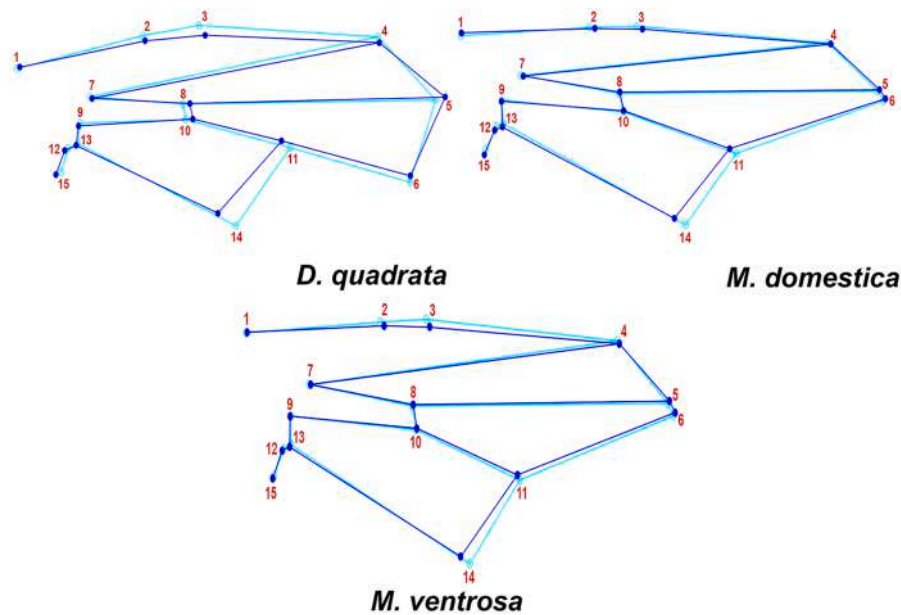


Fig. 6. The wireframe graph showing the differences in the mean wing shape between males (dark blue) and females (light blue) of three muscid fly species obtained from DFA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species, and sex have no correlation with size. Analysis of allometry among six muscid species in this study indicated that wing size elucidated 1.60% of the total shape variation. Although a significant, but weak correlation, between wing size and shape was found, implying that allometry was not a major factor regarding shape differences among species. Of the six analyzed species, significant allometric effects within species were detected only in *H. chalcogaster* and *M. ventrosa*, indicating that size-related shape changes varied among individuals within these two species. Intraspecific allometric effects have been documented in several studies (de Camargo et al., 2015; Jaramillo-O et al., 2015; Oliveira-Christe et al., 2020). Additionally, significant allometric effects were not found between sexes of the three analyzed species (*D. quadrata*, *M. domestica*, and *M. ventrosa*), indicating that allometry took no important role in SSHD. This finding is consistent with previous reports, such as moths (de Camargo et al., 2015) or mosquitoes (Virginio et al., 2015), but contrasting with fruit fly, *Drosophila melanogaster* subgroup (Gidaszewski et al., 2009), which noticed that allometry was a main component of SSHD.

In conclusion, our results demonstrated that a landmark-based geometric morphometric analysis of wings can be applied for genus, species, and sex discrimination of medically and forensically important non-biting muscid flies. This method is easy to use, relatively low-cost, and trustworthy, which can be used as a practical alternative method for species and sex discrimination, particularly when lacking taxonomic experts, and can be performed in the field without complicated equipment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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