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Comparative analysis of the internal and external outlines of wings for an outline-based geometric morphometric approach to distinguish three *Aedes* mosquitoes (Diptera: Culicidae) in Thailand

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Abstract

Aedes mosquitoes belong to a subgenus of Stegomyia (Diptera: Culicidae), which are dengue and chigunkunya vectors, including *Aedes aegypti*, *Ae. albopictus*, and *Ae. scutellaris,* and have a worldwide distribution in tropical and subtropical regions. Species identification of mosquitoes is challenging yet necessary to select appropriate control methods for each species. In this study, we have analyzed the different wing components for identification to find the optimal outline for *Aedes* mosquito analysis by an outline-based GM approach. Two internal and external outlines of the wing were considered for *Aedes* species identification in this study. A total of 90 *Aedes* wings were analysed, divided into 30 wings per species (*Ae. aegypti, Ae. albopictus,* and *Ae. scutellaris*). The results showed that the mean size of *Ae. albopictus* had the largest wings, followed by *Ae. Aegypti,* and *Ae. scutellaris*, respectively, in all the internal and external outlines. While, the shape analysis in the three outlines were expressed by the factor map, the statistical difference and cross-validated classification scores indicated the ability of the outline-based GM approach to distinguish species of *Aedes* mosquitoes. The external outline has a relatively good range of cross-validated classification scores compared to the other internal outlines. The results of this research reveal the suitability of this external outline to identify species of *Aedes* vectors in Thailand.

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Introduction

Mosquitoes are one of medically important insects that transmit human pathogens, such as viruses, protozoans, and nematodes (World Health Organization, 2016). *Aedes* mosquitoes belong to a subgenus of Stegomyia (Diptera: Culicidae), which are dengue and chigunkunya vectors, including *Aedes aegypti*, *Ae. albopictus*, and *Ae. scutellaris,* and have a worldwide distribution in tropical and subtropical regions (Service, 2008; Honório & Castro, 2009). Dengue and chigunkunya are mosquito-borne viral infections, and about half of the world's population is now at risk, particularly in urban and semi-urban areas (Lee *et al*., 2018; Leta *et al*., 2018). For control of these diseases, the World Health Organization has identified that it is relevant to control the *Aedes* populations in endemic areas (World Health Organization, 2014; 2016).

Species identification of mosquitoes is challenging yet necessary to select appropriate control methods for each species (Chaiphongpachara & Sumruayphol, 2017). *Ae. aegypti* and *Ae. albopictus* are primary vectors of the dengue, chigunkunya, and Zika fever viruses, which can be easily be separated for adults based on their morphological features, by observing the patterns on the thorax (Sumruayphol *et al*., 2016). The scutum in the thorax of the *Ae. aegypti* has lyre-shaped white markings, while the *Ae. albopictus* has a long median-longitudinal white stripe. However, in some cases, the specimen is damaged, especially in the thorax, which may lead to errors in the identification of both *Aedes* species (Rattanarithikul *et al*., 2005). *Ae. scutellaris* are reported to be dengue vectors in Papua New Guinea, but not as prominent as *Ae. aegypti* and *Ae. albopictus* (Sumruayphol *et al*., 2016). In addition, the morphological characteristics of *Ae. scutellaris* and *Ae. albopictus* are very similar (Rattanarithikul *et al*., 2010).

Geometric morphometrics (GM) are an alternative method for species identification based on geometry, especially in mosquitoes and others insects (Dujardin, 2008; Lorenz *et al*., 2017). The landmark-based approach is one GM technique which is becoming popular as it is convenient and fast to analyse (Chaiphongpachara, 2018; Chaiphongpachara *et al*., 2018). An outline-based approach is also one of GM techniques that uses the analysis of the contour of forms based on elliptic Fourier analysis that are studied less than the landmark-based approach (Albutra *et al*., 2012; Santillán-Guayasamín *et al*., 2017). Recently, the outline-based GM technique has been used to identify *Aedes aegypti*, *Ae. albopictus*, and *Ae. scutellaris* in Thailand by the external outline and it was found that it is highly effective to distinguish each species (Sumruayphol *et al*., 2016). However, in addition to the external outline, other internal outlines in the wings are interesting for studying the specific location of *Aedes* species identification via an outline-based GM analysis.

In this study, we have analysed the different wing components for identification to find the optimal outline for *Aedes* mosquitoes in the analysis by outline-based GM approach. The results of this study can be used to fulfil to the knowledge of using outline-based GM approach to solve the problem of identification of mosquitoes further.

Materials and Methods

Aedes mosquito collection

The larvae and pupae stage of the three *Aedes* species were collected by a dipping method in water containers in the communities of the Mueang Samut Songkhram District and Samut Songkhram Province, Thailand, during August 2018. Mosquito samples were transported and reared in a laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University. The conditions of the laboratory in this study was controlled at 25°C and 50-60% relative humidity under a 12/12 h light/dark cycle. While rearing mosquito larvae, plastic trays containing filtered water were used and provided 0.1 g of dog food per day. When the larvae stage developed into pupae, they were transferred to 30×30×30 cm cages to facilitate emergence. After that, the adult female *Aedes* mosquitoes that emerged were morphologically identified using taxonomy (Rattanarithikul *et al*., 2005; 2006).

Mosquito wing preparation

Thirty individuals in each species of female *Aedes* mosquitoes were randomly selected using a table of random numbers. The right wing of each mosquito was used in this study for GM analysis by dissection and mounting on glass microscope slides with a coverslip using Hoyer's medium. All slides were photographed using a Nikon DS-Ri1 SIGHT digital camera connected to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) under 40× magnification with a 1 mm scale bar included in all wing pictures.

Outline-based GM analysis

Two internal and one external outlines of the wing in Figure 1 were considered for *Aedes* species identification. In this research, a repeatability test was implemented for evaluating the accuracy of the measurement marked before GM analysis (Arnqvist & Märtensson, 1998).

The size was estimated as perimeter of each outline, while the elliptic Fourier analysis was used to analyze shape variables. Wing size variation between *Aedes* species in each internal and external outline was illustrated by quantile boxes, while the wing shape variation was illustrated by the superimposition of the mean forms. A factor map was created to show shape divergence for each species from a discriminant analysis. In addition, discriminant analysis also calculated Mahalanobis distance, which is used to determine the variance and correlation of variables.

Statistical significance of the perimeter of the outline and the Mahalanobis distance of the internal and external outlines between *Aedes* species in each pair was calculated by 1000 runs of non-parametric permutation tests with a Bonferroni correction at P-values <0.05. Finally, a cross-validated classification test was used to show percent accuracy between species.

Software

The CLIC (Collection of Landmarks for Identification and Characterization) package, version 97 (Dujardin & Slice, 2007), which is freely available at https://xyom-clic.eu, was used for outline-based GM analysis.

Results

A total of 90 *Aedes* wings were analyzed, divided into 30 wings per species (*Ae. aegypti, Ae. albopictus,* and *Ae. scutellaris*). The results of the repeatability test of the size and shape to determine the accuracy of measurement repeatedly showed good quality in each internal and external outline. The measurement error for

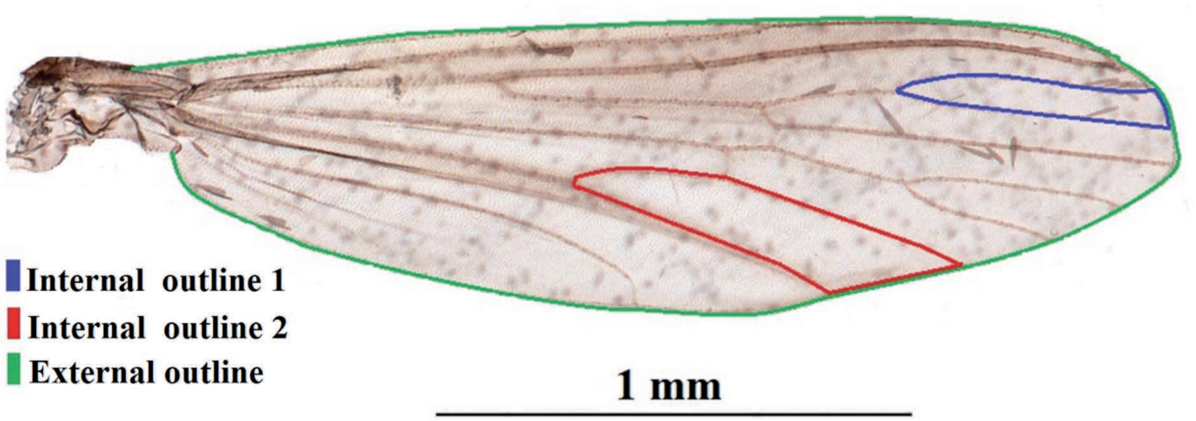

Figure 1. Three outlines digitized for the wing of *Aedes* mosquito. Each outline for outline-based GM analysis included an internal outline 1 (blue), internal outline 2 (red), and external outline (green). Size was estimated as the perimeter of the outline.

size and shape was <1% (for repeatability of size, it was 0.96 and for shape, it was 0.94).

Wing size analysis

The wing size of each species of *Aedes* mosquitoes was estimated by the perimeter of the outline. Variation of the wing perimeter between species in each internal and external outline was shown in Figure 2. The mean size of *Ae. albopictus* wings was the largest, followed by *Ae. aegypti,* and *Ae. scutellaris*, respectively, in all the internal and external outlines. Statistical comparisons of the mean perimeter of the internal and external outlines of the wing between *Aedes* species indicated the difference in some pairwise, including that *Ae. scutellaris* was different from *Ae. aegypti* and *Ae. albopictus* in the internal outline 1 and the external outline of the *Aedes* wing, while *Ae. albopictus* was different from *Ae. aegypti* and *Ae. scutellaris* in internal outline 2 (P<0.05; Table 1).

Shape analysis

A discriminant evaluation analysis variation on wing shape among species was derived after the superimposition algorithm of the mean outlines of *Aedes* species in each internal and external outline of the wing (Figure 3). Discriminant analysis was taken to create factor maps to visualize, which found that they have little overlap in the external outline (Figure 4). Every pairwise Mahalanobis distance has statistical differences in internal outline 1 and external outline (P<0.05), while the pairwise of *Ae. albopictus* and *Ae. scutellaris* did not show a statistical difference in internal outline 2 (P>0.05; Table 2).

Table 1. Statistical comparisons of the perimeter of the internal and external outlines of the *Aedes* wing between species.

Species	Mean±Standard deviation (mm) of the perimeter		
	Internal outline 1	Internal outline 2	External outline
Ae. aegypti	1.43±0.02^{a}	1.93±0.02^{a}	5.32-0.21^{a}
Ae. albopictus	1.45±0.01^{a}	2.06±0.03^{b}	5.39-0.16^{a}
Ae. scutellaris	1.25±0.03^{b}	1.90±0.06^{a}	4.92-0.32^{b}

In each row, different superscript letters are statistical differences at P<0.05.

Table 2. Statistical significance of pairwise Mahalanobis distance between *Aedes* species in each internal and external wing outline.

Outlines	Species	*Ae. aegypti*	*Ae. albopictus*	*Ae. scutellaris*
Internal outline 1	*Ae. aegypti*	-		
	Ae. albopictus	3.62*	-	
	Ae. scutellaris	3.04*	4.88*	-
Internal outline 2	*Ae. aegypti*	-		
	Ae. albopictus	4.43*	-	
	Ae. scutellaris	4.49*	3.44	-
External outline	*Ae. aegypti*	-		
	Ae. albopictus	2.65*	-	
	Ae. scutellaris	3.57*	3.05*	-

*Statistical differences.

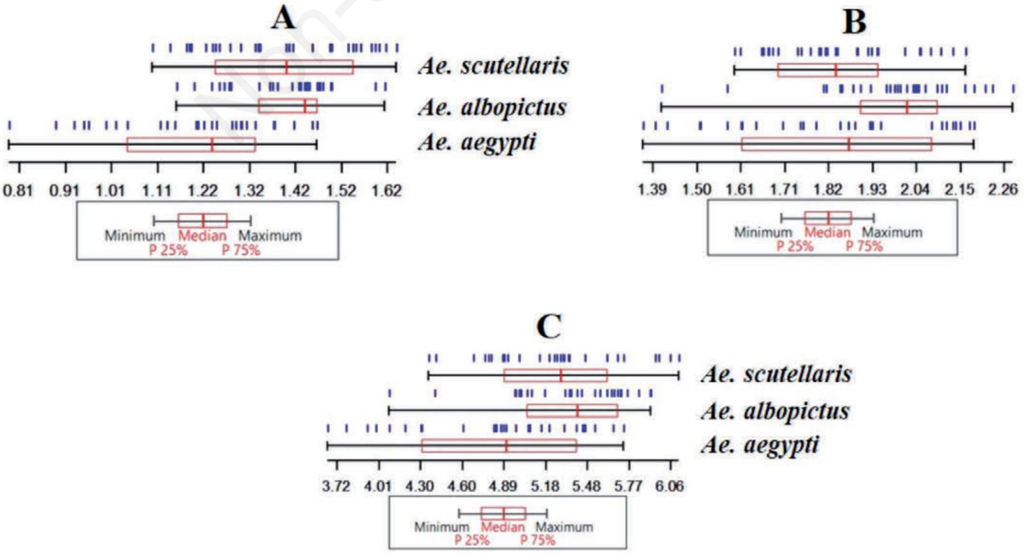

Figure 2. Variation of wing perimeter (mm) of each species of *Aedes* mosquitoes (A=internal outline 1, B=internal outline 2, and C=external outline). Each box shows the group median that separates the 25th and 75th quartiles, and vertical bars represent the sizes of the individual *Aedes* sample.

A cross-validated classification test revealed the analytic capabilities of all three outlines by outline-based GM analysis. The range of accuracy in the *Aedes* species classification of the internal outline 1 was 46-73%, of the internal outline 2 was 40-63%, and of the external outline was 53-70% (Table 3).

Discussion and Conclusions

In this study, we evaluated the internal and external outlines of wings in mosquitoes for an appropriate position of the outline-based GM technique in the discrimination of three *Aedes* species. The results of the study revealed different efficiencies in each outline, including size and shape, when used for the outline-based GM analysis with *Aedes* mosquitoes.

Wing size

The mean of the perimeter of outlines among *Aedes* mosquetoes were similar in all different outlines (*Ae. albopictus* > *Ae. aegypti* > *Ae. scutellaris),* which shows the relationship between each outline with the wing. The statistical tests found that each outline had a difference in each pairwise of different species. This underscores the importance of the outline part in the outline-based analysis, which affects the size results. This result of external outline analysis was consistent with a previous study that identified species of *Aedes* mosquitoes in Thailand (Sumruayphol *et al*., 2016). However, shape is not widely used in the classification of organisms as they are highly variable from the effects of the environment, such as temperature and quality of food received in the habitat (Jirakanjanakit *et al*., 2007; Dujardin, 2008; 2011; Kaba *et al*., 2017).

Wing shape

For species identification, shape is considered more appropriate than size factors. The results of the shape factor analysis in the three outlines were expressed by the factor map, statistical difference, and cross-validated classification scores that indicate the ability of an outline-based GM approach to distinguish species of *Aedes* mosquitoes. These are in line with previous research that used outline-based GM to identify mosquito vectors of different species in Ratchaburi Province, Thailand, which can separate *Ae. aegypti* and *Ae. albopictus* from other mosquitoes within the range of 86% and 95%, respectively (Chaiphongpachara, 2018). Cross-validated classification scores revealed that each outline has different efficacy in identifying *Aedes* mosquitoes, especially in *Ae. aegypti,* including 46% of internal outline 1, 50% of internal outline 2, and 66% of external outline. This was different from the study of Sumruayphol *et al.,* (2016), which found that *Ae. aegypti* were best classified by the outline method (87% for external outline analysis).

However, this inconsistency may be due to morphological variations because of the various environments in each area. An external outline has a relatively good range of cross-validated classification scores compared to other internal outlines [53-70% (external outline) *vs* 46-73% (internal outline 2) and 40-63% (internal outline 1)]. It is very important to use the outline as a location for identification by an outline-based GM approach. The results of this research reveal the suitability of this external outline to identify the species of *Aedes* mosquitoes as dengue, chigunkunya, and Zika vectors.

An outline-based GM technique is one approach of GM that can help in the identification of some species of mosquitoes effectively, such as separating *Ae. scutellaris* from *Ae. aegypti* and *Ae. albopictus*. The outline for the analysis is very important in this approach, which must be specific to the mosquito species used in the identification. The results of this study support that the external outline has the ability to be analyzed by an outline-based GM technique for *Aedes* mosquitoes better than other internal outlines.

Table 3. Cross-validated classification of three species of *Aedes* mosquitoes in each internal and external wing outline.

Species	Percent accuracy of classification (assigned/observed)		
	Internal outline 1	Internal outline 2	External outline
Ae. aegypti	46 (14/30)	50 (15/30)	66 (20/30)
Ae. albopictus	53 (16/30)	40 (12/30)	53 (16/30)
Ae. scutellaris	73 (22/30)	63 (19/30)	70 (21/30)

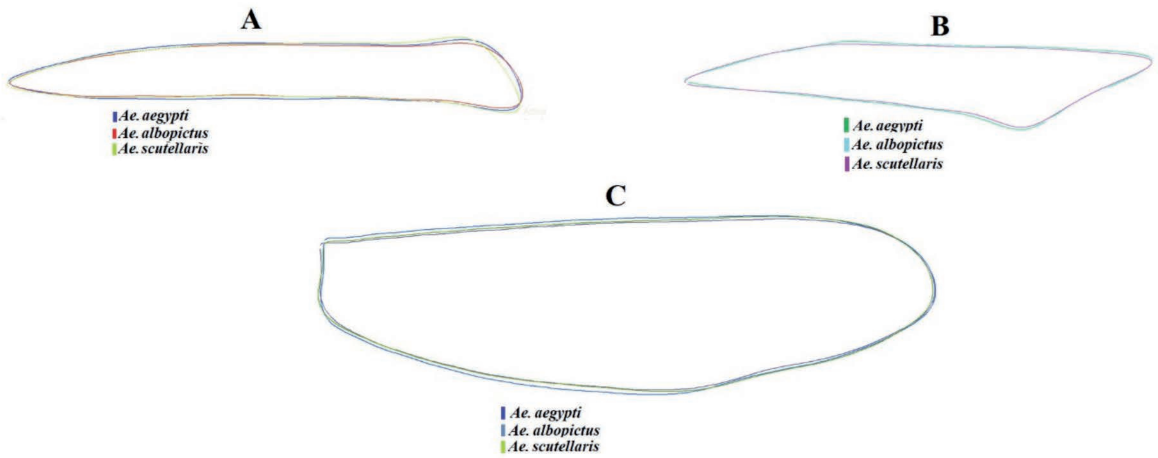

Figure 3. Superimposition of mean outlines of each *Aedes* species. (A=internal outline 1, B=internal outline 2, and C=external outline).

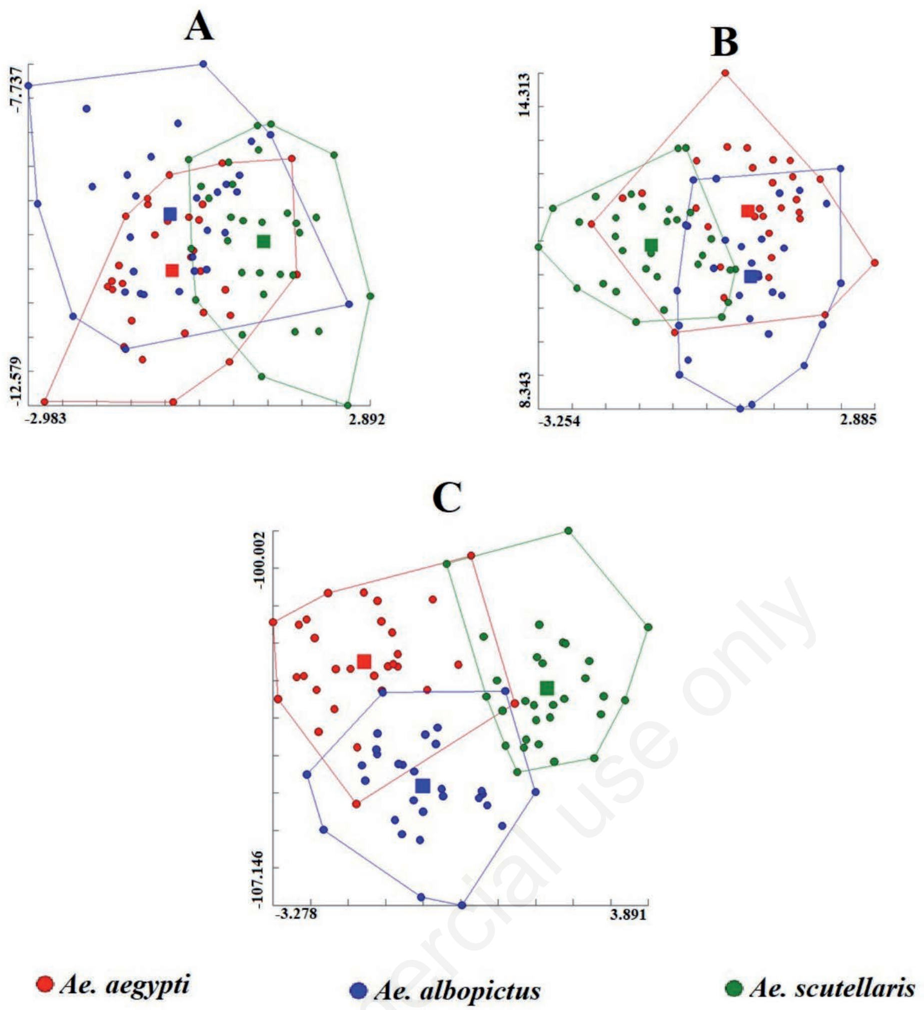

Figure 4. Factor maps from discriminant analyses showing the variation of wing shape in *Ae. aegypti* (red), *Ae. albopictus* (blue), and *Ae. scutellaris* (green) in each internal (cell 1 [A] and cell 2 [B]), and external outline (C).

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