

Short Communication: Landmark-based geometric morphometric analysis of wings to distinguish the sex of *Aedes* mosquito vectors in Thailand

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Abstract. Chaiphongpachara T, Laojun S. 2019. Short Communication: Landmark-based geometric morphometric analysis of wings to distinguish the sex of *Aedes* mosquito vectors in Thailand. *Biodiversitas* 20: 419-424. *Aedes* mosquitoes (Diptera: Culicidae) are medically important insects which are vectors of yellow fever, dengue fever, chikungunya, West Nile, and the Zika virus, emerging problems worldwide. Typically, male (non-vector) and female (vector) *Aedes* mosquitoes can easily be separated, however, the samples in the field is often incomplete, making it difficult to separate male and female mosquitoes. The goal of this research is to study the effectiveness of the landmark-based geometric morphometric technique to distinguish the sex of male and female *Aedes* mosquito vectors, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*, in Thailand. Evaluation of wing size by centroid size analysis found that males and females are distinctly different; females are larger than males in three species of *Aedes* mosquito. The wing centroid size of *Ae. aegypti* and *Ae. albopictus* were very similar, however, *Ae. scutellaris* was smaller than in both other species. The wing shape between sexes was different in all groups of *Aedes* mosquitoes. The accuracy of the sex's classification of *Aedes* vectors was quite high (more than > 80% from the cross-validated reclassification test). The results of this study prove that landmark-based geometric morphometric can distinguish sexes in *Aedes* vectors which can be used to solve problems in the field when it is necessary to distinguish the sexes of *Aedes* mosquitoes with damaged samples.

Keywords: Landmark-based geometric morphometric analysis, *Aedes* mosquito, vectors, Thailand

INTRODUCTION

Aedes mosquitoes (Diptera: Culicidae) are medically important insects and are vectors of yellow fever, dengue fever, chikungunya, West Nile, and the Zika virus, emerging problems worldwide (Service 2008; World Health Organization 2016). In addition, *Aedes* species were reported in some areas where they can be vectors of *Wuchereria bancrofti* and *Brugia malayi* as filarial nematodes (Service 2008). *Aedes aegypti* (Linnaeus) and *Ae. albopictus* (Skuse) have been considered important vectors of many other arboviruses (Kraemer et al. 2015). *Ae. aegypti* is a native mosquito in sub-Saharan Africa, while *Ae. albopictus* is native to southeast Asia (Kraemer et al. 2015). Currently, *Ae. aegypti* have spread globally to tropical and sub-tropical regions of the world, while *Ae. albopictus* has spread to islands in the Indian and Pacific Oceans (Leta et al. 2018). The distribution of them increases the risk occurrence of *Aedes*-borne viral diseases to humans. Diseases such as dengue, Zika, and chikungunya have increased in many areas (Gardner et al. 2017; Vega-Rua et al. 2014).

Thailand is one country affected by mosquito-borne diseases epidemically, especially dengue fever (Chaiphongpachara T 2017; Clark et al. 2005). In 2017, there were 31,843 cases of dengue fever, and its morbidity rate was 48.20 per 100,000, while the mortality rate was

0.01 per 100,000 (Ministry of Public Health, Thailand 2017). In Thailand, there are two species of *Aedes* mosquito, including *Ae. aegypti* and *Ae. albopictus*, that are medically important and very common. *Ae. scutellaris* (Walker) is a species of *Aedes* which was found in sympatry with both *Ae. albopictus* and *Ae. aegypti* (Rattarithikul et al. 2005; Rattarithikul et al. 2010). While, this species is not reported as a vector, it is still unclear in Thailand how to separate it from *Ae. albopictus* because of their similar morphology.

At present, research on mosquito-borne diseases is rapidly developing to stop outbreaks, such as the development of vaccines (Schwartz et al. 2015), methods of mosquito control (Chaiphongpachara et al. 2018; Chaiphongpachara et al. 2018), and genetic modification of mosquitoes to reduce the number of vectors in nature (Terenius et al. 2008). Reducing the number of mosquitoes in areas at risk is another way to control mosquito-borne diseases (Benelli et al. 2016). However, the first step in successful mosquito control is to know the species of mosquito vectors in a particular area as the specific behaviour of each species is key to effective control.

Recently, the geometric morphometric (GM) technique has become a popular method for the identification of similar morphological species or species complex of mosquitoes in many countries (Dujardin 2011; Lorenz et al. 2017). It also helps solve the problem of identification of

mosquitoes collected in the field as the sample is often incomplete, making it difficult to identify (Chaiphongpachara 2018). Advantages of this technique are easy to use, fast, and affordable (Rohlf 2002). For *Aedes* mosquitoes, the GM technique has been used in Thailand to identify species, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*, and it can separate them from each other (Sumruayphol et al. 2016). Although species identification of *Aedes* mosquitoes by GM succeeded, using it to distinguish the sexes is still unclear. Typically, male (non-vector) and female (vector) mosquitoes can be easily separated, however in the field, it is difficult to classify the sex if the samples are damaged.

Thus, the goal of this research is to study the effectiveness of the landmark-based GM technique in Thailand to distinguish the sex between male and female *Aedes* mosquito vectors, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*. It is useful and important to the field of mosquito vector studies to separate mosquitoes by vector and not vector when the *Aedes* sample is incomplete.

MATERIALS AND METHODS

Mosquito collections

Three species of *Aedes* mosquitoes, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris* were collected by dipping immature larvae and pupae stage samples in the area of the Samut Songkhram Province. Samut Songkhram Province was selected as a sample collection area due to the epidemic of dengue fever in this area of Thailand (Chaiphongpachara et al. 2017). Containers in the houses in the Mueang Samut Songkhram District have been surveyed and the larvae were collected in August 2018. The larvae and pupae of the *Aedes* mosquito samples were

reared in the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Center, Thailand. Plastic trays containing filtered water were used to rear the mosquito larvae and they were provided dog food once a day. When they developed into adult mosquitoes, they were identified as *Aedes* species using taxonomic keys based on their morphology (Rattanaarithikul et al. 2005; Rattanaarithikul et al. 2010).

Wing preparation

Sixty individual per species of *Aedes* mosquitoes (30 male and 30 female) were used for the GM analysis. Only right wings of the *Aedes* mosquito samples were dissected and mounted on glass microscope slides with a 0.08-0.12 mm glass coverslip using Hoyer's mounting medium. After that, *Aedes* wing samples were photographed using a digital camera connected to a Nikon SMZ745T stereo-microscope (Nikon Corp., Tokyo, Japan).

Landmark-based GM approach

In this study, CLIC (Collection of Landmarks for Identification and Characterization) software, which is freely available at <https://xyom-clic.eu> was used for landmark-based GM analyses. Seventeen landmarks (LM) on the wings of each *Aedes* individual were selected and digitized, as shown in Figure 1. Before GM analysis, all samples in each species of *Aedes* mosquitoes were digitized twice to compare with the first digitized images for measurement error, which was estimated by a repeatability index (Arnqvist and Mårtensson 1998). For the analysis of GM, raw coordinates of wing samples in each group were superimposed using Generalized Procrustes Analysis (GPA) (Rohlf 1990). This procedure separates size (centroid size) and shape (partial warps [PW]) variables.

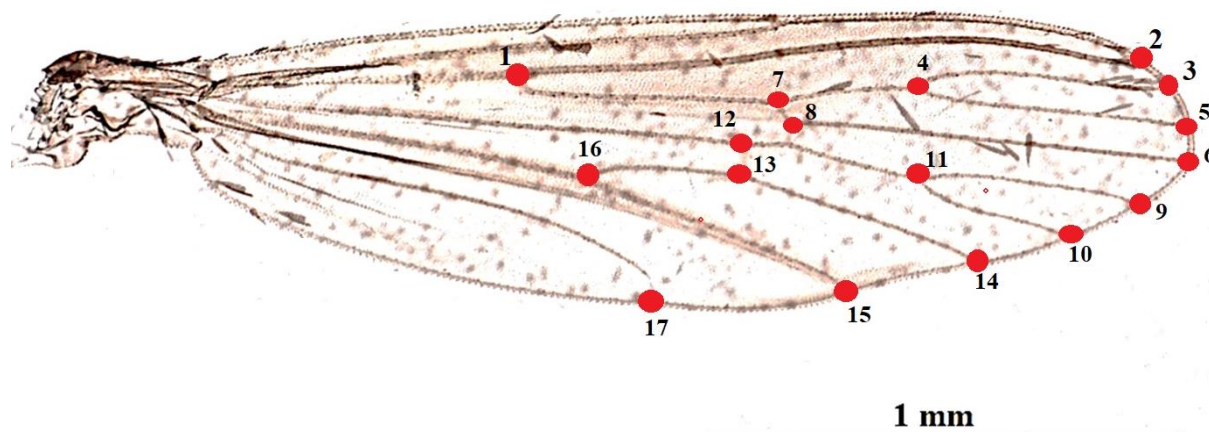


Figure 1. Seventeen LMs on the wing of an *Aedes* mosquito for GM analyses

Size variables

The wing size estimation in male and female *Aedes* mosquitoes used centroid size (CS), which is defined as the square root of the sum of the squared distances from the centroid to the individual in each landmark (Dujardin 2011). Quantile boxes separating the 25th and 75th quartiles median scores were shown in each box and were created to visualize the CS variations in the males and females of each *Aedes* species. The statistical significance of average CS difference between males and females in each species was assessed using a non-parametric permutation test (1,000 cycles). A Bonferroni correction was corrected, indicating a statistical significance at p -value < 0.05 .

Shape variables

After GPA, PW as wing shape variables was computed for shape comparison between males and females in each species. The principal components of the PW (or relative warps) were used as input for the discriminant analyses (DA) or canonical variate analysis (CVA), which were used to calculate the factor map for females and males, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*. Then, Mahalanobis distance between sexes was calculated from DA analysis and the difference in each pair was tested using a non-parametric permutation test (1,000 cycles) with a Bonferroni correction at p -value < 0.05 . Percent accuracy in the separation between males and females used a cross-validated reclassification test based on the Mahalanobis distance. Finally, a Neighbor Joining tree was created based on Procrustes distances to illustrate morphological divergence between females and males in *Aedes* species.

RESULTS AND DISCUSSION

A total of 180 *Aedes* wings for GM study were divided into 60 wings per species, including 30 male and 30 females for each: *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*. For this study, we tested the accuracy of seventeen LMs repeatedly marked by a single operator. The results of the measurement error showed that there were good scores for size and shape in the three *Aedes* species, including 0.98 and 0.96 for size and shape of *Ae. aegypti*, 0.95 and 0.96 for size and shape of *Ae. albopictus*, and 0.93 and 0.97 for size and shape of *Ae. scutellaris*.

Wing size

There were wing CS variations between the sexes of the three *Aedes* species, as shown in Figure 2. In comparison of wing CS between males and females, the wings of females were significantly higher than those of males in all species of *Aedes* ($p < 0.01$; Table 1). The mean wing CS of *Ae. aegypti* was very similar, with *Ae. albopictus* in both sexes (2.25 vs. 2.29 in the female and 1.84 vs. 1.83 in the male, respectively). *Ae. scutellaris* was smaller than *Ae. aegypti* and *Ae. albopictus* in both sexes (Table 1).

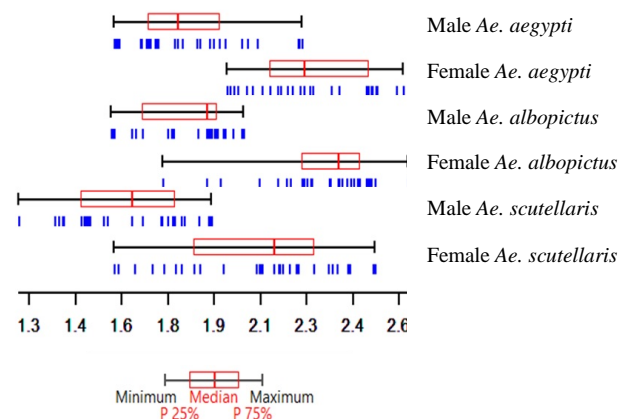


Figure 2. Wing CS variations between sexes (in mm), were shown as quantile boxes in which each quantile box shows the median scores separating 25th and 75th quartiles

Table 1. Mean wing CS differences between males and females in each *Aedes* species

<i>Aedes</i> mosquito	Mean±SD (mm)	Max-Min (mm)	p -value
Male <i>Ae. aegypti</i>	1.84±0.04	2.23-1.59	< 0.01*
Female <i>Ae. aegypti</i>	2.25±0.03	2.58-1.97	
Male <i>Ae. albopictus</i>	1.83±0.02	2.03-1.58	< 0.01*
Female <i>Ae. albopictus</i>	2.29±0.03	2.59-1.75	
Male <i>Ae. scutellaris</i>	1.62±0.04	1.91-1.26	< 0.01*
Female <i>Ae. scutellaris</i>	2.08±0.07	2.48-1.58	

Note: * = significant differences at $p < 0.05$

Wing shape

The mean residual coordinates of both males and females in each *Aedes* species were superimposed onto the consensus residual coordinates, displaying patterns of shape variation for comparing differences of wing venation between the sexes, as shown in Figure 3. Morphospaces based on wing shape variables from a principal component and factor map of the discriminant factor indicate the differences clearly. The morphospaces found little overlap in male and female groups in the three species (Figures 4A, B, and C). While in the factor map, an overlap between the two groups was not found. The highest Mahalanobis distance score (Table 2) is 9.95 of male and female *Ae. scutellaris*, followed by *Ae. albopictus* (8.16), and *Ae. aegypti* (5.43) (The higher the value, the greater the difference of wing shape).

A non-parametric permutation test with a Bonferroni correction test showed statistically significant differences between the sexes in each species ($p < 0.01$). Percent accuracy for sex separation by a cross-validated reclassification test showed quite a high value, providing a mean correct assignment of more than 80% in all species. Finally, the Neighbor Joining tree based on Procrustes distances of both males and females also confirmed the differences in wing shape between sexes, in which males and females are distinctly separated (Figure 5).

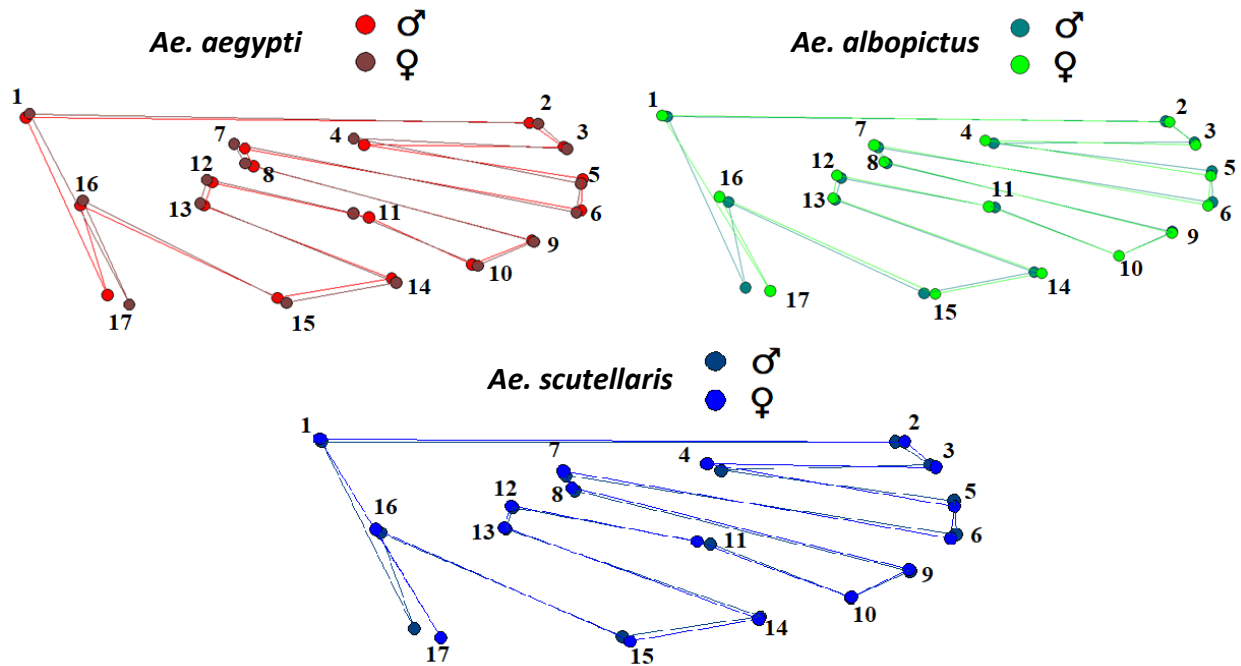


Figure 3. Shape variations between sexes after Procrustes superimposition of 17 landmarks which mean of lines are connecting with landmarks. (red = *Ae. aegypti*, green = *Ae. albopictus*, and blue = *Ae. scutellaris*)

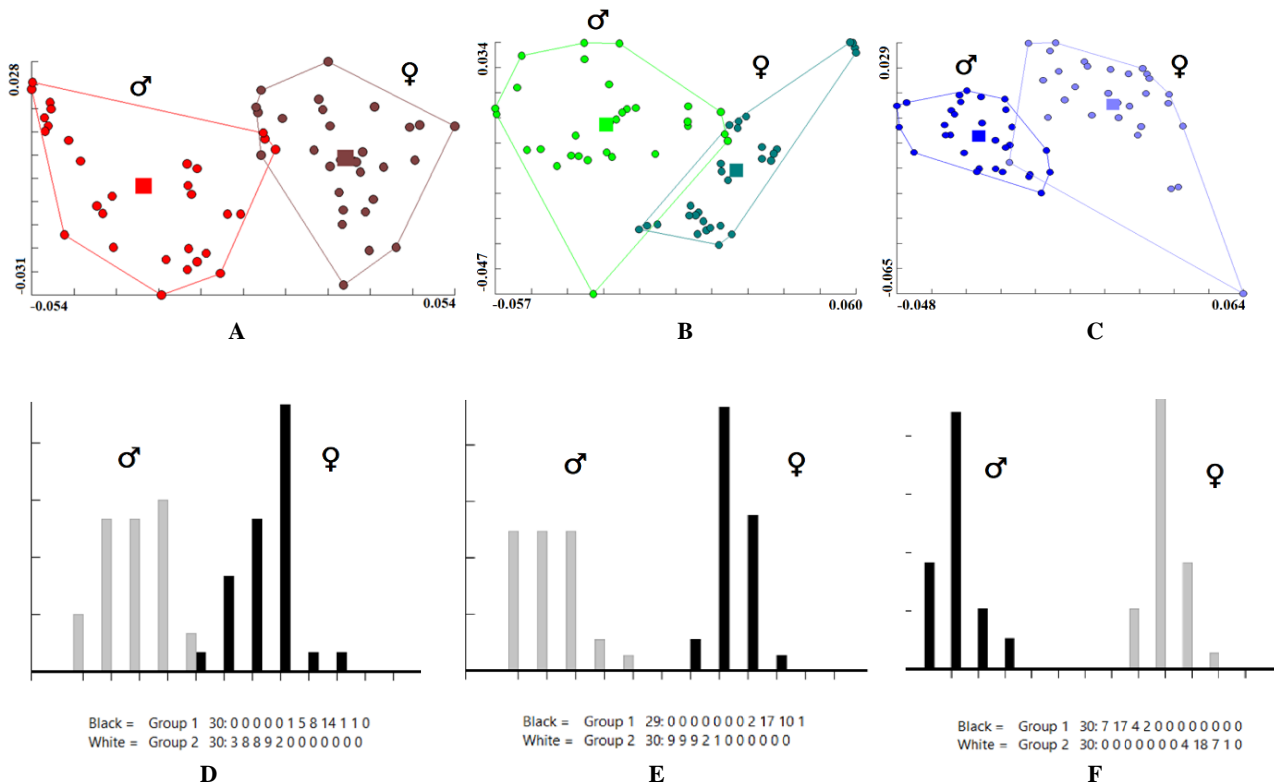


Figure 4. Morphospaces of both male and female mosquitoes based on wing shape variables from principal component, as shown A. *Ae. aegypti*., B. *Ae. albopictus*., and C. *Ae. scutellaris*.. While, D. *Ae. aegypti*., E. *Ae. albopictus*., and F. *Ae. scutellaris* were factor maps of the discriminant factor separating males (grey bars) and females (black bars)

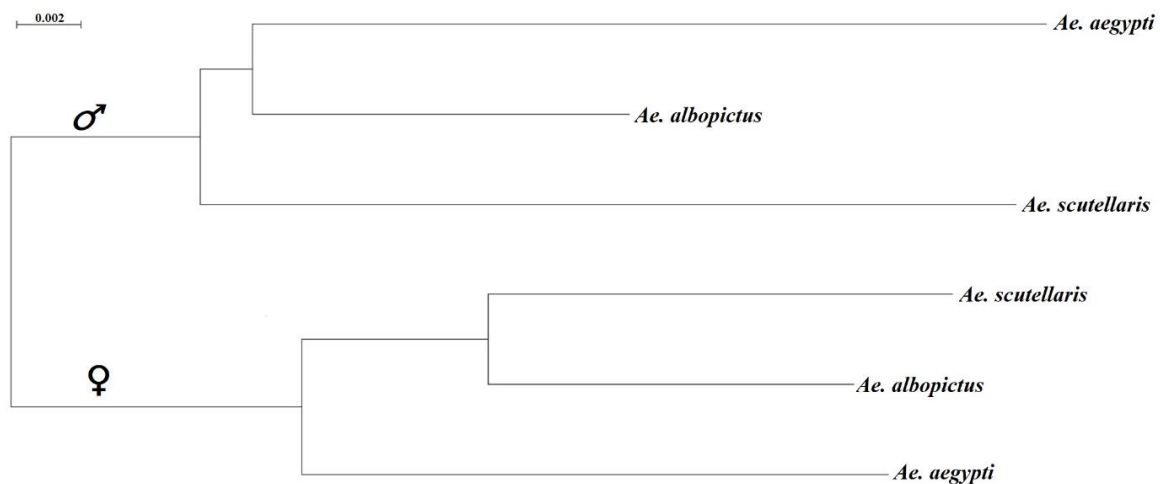


Figure 5. Neighbor joining tree based on Procrustes distances of males and females of three *Aedes* species

Table 2. Mahalanobis distances between sexes and cross-validated reclassification scores based on wing shape of *Aedes* mosquito

<i>Aedes</i> mosquito	Mahalanobis distances scores	<i>p</i> -value	Percent accuracy in separation (assigned/observed)
Male <i>Ae. aegypti</i>	5.43	< 0.01	83 (25/30)
Female <i>Ae. aegypti</i>			90 (27/30)
Male <i>Ae. albopictus</i>	8.16	< 0.01	100 (100/30)
Female <i>Ae. albopictus</i>			86 (26/30)
Male <i>Ae. scutellaris</i>	9.95	< 0.01	93 (28/30)
Female <i>Ae. scutellaris</i>			93 (28/30)

Discussion

The sex classification of mosquitoes is initially important for species identification. Typically, in medical entomology, studies focus on mosquito vectors in each area endemic for mosquito-borne diseases (Chaiphongpachara and Sumruayphol 2017). Female mosquitoes are vectors of insect-borne diseases because they must bite humans or animal and feed on the blood to obtain protein and necessary nutrients for the development of their eggs (Killick-Kendrick 1996). Male mosquitoes are not vectors, as they cannot bite but instead feed on nectar and other naturally occurring sugary secretions (Service 1993). The difference between males and females is often observed in the form of the antennae, including the plumose in males, which has long hairs and the pilose in females with a few short hairs (Rattananarithikul et al. 2005). However, if this vital organ is damaged or has disappeared, it may cause a barrier to separate vectors (females) and non-vectors (males). Our results on the landmark-based GM analysis in *Aedes* wings to distinguish sexes revealed the high efficiency of separation in three *Aedes* mosquito, including *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris*.

An evaluation of wing size by CS analysis found that males and females are distinctly different; females are

larger than males in three species of the *Aedes* mosquito. The wing CS of *Ae. aegypti* and *Ae. albopictus* were very similar, but *Ae. scutellaris* was smaller than both species. This is in line with previous research that has examined species identification of *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris* in Thailand by GM techniques and found size differences in each species where *Ae. scutellaris* was the smallest (Sumruayphol et al. 2016). Typically, the identification of mosquito spp. by GM does not use size factors to join in the decision because a variation of size can easily happen from the environment (Sumruayphol et al. 2016; Chaiphongpachara 2018; Wilke et al. 2016). However, for gender discrimination, size factors are important factors that must be used to classify sex.

The wing shape between males and females was different in all groups of *Aedes* mosquitoes, which corresponds to the results of the wing sizes. Mahalanobis distance value indicates the scores of morphological differences between the sexes in the three *Aedes* species. *Ae. scutellaris* had the most differences, followed by *Ae. albopictus* and *Ae. aegypti*, respectively. The accuracy of the sex classification of *Aedes* vectors was quite high (more than > 80% from a cross-validated reclassification test). In addition, the Neighbor Joining tree showed groupings between sexes, clearly indicating that both males and females were very different in their morphology, which supports the statistical difference by the non-parametric permutation test (1,000 cycles) with a Bonferroni correction test ($p < 0.01$).

Wing venation is a unique identity that distinguishes the species of mosquitoes (Wilke et al. 2016). This is consistent with previously research showing that this identity can be used to distinguish mosquito species complex which has a very similar morphology in their group (Sumruayphol et al. 2016). The results of this study prove that landmark-based GM can distinguish sexes in *Aedes* vectors, which can be used to solve problems of gender discrimination in the field further.

In conclusions, GM is one of techniques used for solving the problem of species identification and studying variations in populations of mosquito vectors. This study confirms the efficacy of GM for sex classification and supports the application of this technique in the field. In addition, this technique is very interesting and as easy to use, fast, and inexpensive.

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