

Using Landmark and Semi-Landmark-Based Geometric Morphometric to Identify Three Species of *Aedes* Mosquitoes in Thailand

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Abstract: Geometric Morphometrics (GM) is a new effective technique for mosquito identification and has become popular in applications to entomological work. In this study, we evaluated the Landmark and Semi-Landmark-based approach (LMSL) of GM to distinguish three species of *Aedes* mosquitoes in Thailand, including *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris* as medically important vectors and compared the efficacy of LMSL and the Landmark-based approach (LM) to investigate both of their potentials. We were collected *Aedes* populations at the larval and pupal stages in human-made water containers in the communities of the Mueang Samut Songkhram District, Samut Songkhram Province, Thailand. The results of the comparison between LM and LMSL in this study revealed that they had similar efficacies for three *Aedes* species separations based on shape variation. However, for some *Aedes* species such as *Ae. scutellaris*, LMSL can better separate than LM while *Ae. albopictus* have better separation with LM than LMSL. Therefore, we recommend that before applying this technique, there should be a comparison with other GM methods to determine the specificity of species of mosquito vector in order to find an appropriate GM technique.

Key words: Geometric morphometric, landmark and semi-landmark-based approach, *Aedes aegypti*, *Aedes albopictus*, *Aedes scutellaris*, GM methods

INTRODUCTION

Dengue is still a major problem causing illness and death worldwide, especially in tropical and sub-tropical areas (Chaiphongpachara *et al.*, 2017; Lee *et al.*, 2018). Over the last 50 years, dengue's incidence has risen 30-fold over with an estimated 50-100 million infections per year and more than 100 endemic countries (Guzman *et al.*, 2010; Ebi and Nealon, 2016). Chikungunya is also a major public health problem that mostly occurs in Africa, Asia and the Indian subcontinent (Mahendradas, 2016; Vu *et al.*, 2017). Both dengue and chikungunya are mosquito-borne viral diseases transmitted by *Aedes* mosquitoes (Service, 2008; Chaiphongpachara and Moolrat, 2017). *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris* belong to a subgenus of *Stegomyia* (*Diptera*; *Culicidae*) which are vectors associated with these diseases (Sumruayphol *et al.*, 2016). Typically, these three species of *Aedes* mosquitoes are of the sympatric field. However, they have unique characteristics, including patterns on the thorax such as lyre-shaped white markings for *Ae. aegypti* and a long median-longitudinal white

stripe for *Ae. albopictus* but often fail to distinguish species owing to damage with the *Aedes* specimen, especially, the scutum part (Rattanarithikul *et al.*, 2010). These classification errors may have led to difficulties in planning for *Aedes* mosquito population control in endemic areas. New alternative methods to resolve this problem are needed.

Geometric Morphometric (GM) is a novel, effective technique based on the coordinates of identifiable landmarks that has become popular in applications to entomological work, especially insects of the family, *Culicidae* (Lorenz *et al.*, 2012, 2017; Chaiphongpachara, 2018). The advantages of this technique are that its inexpensive, easy to use and fast, supported by specialized analysis software (Garros and Dujardin, 2013; Dujardin, 2008). Therefore, this procedure is so attractive that even though molecular techniques are powerful but they are expensive. Many species of mosquitoes have numerous similarities in terms of morphology such as species complex, cryptic species, sibling species and morphologically close species, so, sometimes, morphological identification that occurs is faulty (WHO.,

2007). The Landmark-based approach (LM) is the first geometric morphometric method to solve the mosquito classification problem. Wings of mosquitoes are suitable organs for analysis by GM techniques which are bi-dimensional and specific to each species (Wilke *et al.*, 2016). However, the weaknesses of the LM method include that it may not cover certain landmarks at curved positions, unique features of certain species. For mosquitoes in some areas of the wing, there is an important curve that may be species-specific.

The Semi-Landmark-based (LMSL) approach is a GM technique developed from LM combined with semi-landmarks (locations of points on curves) analyses (Kaba *et al.*, 2017). Therefore, in this study, we assessed the LM and LMSL approach of GM to distinguish three species of *Aedes* mosquitoes in Thailand, including *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris*-medically importance vectors. In addition, we were compared the efficacy of LMSL and LM to investigate the potential of both approaches for identifying *Aedes* mosquitoes.

MATERIALS AND METHODS

***Aedes* collection and identification:** In this study, we collected *Aedes* populations at the larval and pupal stages in human-made water containers using a dipper, ladle spoon, dropper and pipette. All sample collection was carried out in the communities of the Mueang Samut Songkhram District, Samut Songkhram Province, Thailand during August 2018. Afterwards, mosquito samples were sent to the College of Allied Health Sciences, Suan Sunandha Rajabhat University for rearing in a laboratory. Controlling the rearing conditions in laboratory was essential and in this study was set as $25 \pm 2^\circ\text{C}$, 50-60% relative humidity and 12:12 h light: dark. Larvae and pupae were reared in plastic trays containing filtered water ($30 \times 40 \times 7$ cm) and were provided 0.1 g of dog food daily. After emergence, pupae were transferred to $30 \times 30 \times 30$ cm cage to wait for the development of the adult stage. Only adult female *Aedes* mosquitoes as vectors (male mosquitoes are not vectors) were identified based on the morphological taxonomic keys (Rattananarithikul *et al.*, 2005, 2010).

Wing preparation and data collection: Female adult *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris* were used in this study. The samples employed were selected from the integrity of the wings without tearing and clear visibility of wing venation. The right wing of each *Aedes* sample was dissected and mounted on microscope slides with a coverslip using Hoyer's medium. Thereafter, *Aedes*

wings on slides were photographed by a Nikon DS-R11 SIGHT digital camera connected to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) at $40\times$ magnification with a scalebar (1 mm) indicated on the photograph.

Geometric morphometric analyses

Landmark-based GM approach (LM): The 11 landmarks in Fig. 1 were digitized and used for LM in this study. After landmark collection, all photographs were repeated and compared to the set used for investigating the quality of landmarks before GM analysis (repeatability test) (Arnqvist and Martensson, 1998).

Size variations between *Aedes* species was estimated by Centroid Size (CS) which is the square root of the sum of the squared distances between the center of the configuration of landmarks and each landmark (Bookstein, 1991). CS variation was created as quantile boxes for comparison between *Aedes* groups while significant differences for CS variation among *Aedes* mosquitoes was based on non-parametric, permutation tests (1000 cycles) with post hoc pair wise Bonferroni correction test at $p < 0.05$.

Shape variable (Partial Warp (PW) scores) of *Aedes* wings were computed from principal components variables from principal components was performed to calculated after generalized Procrustes analysis.

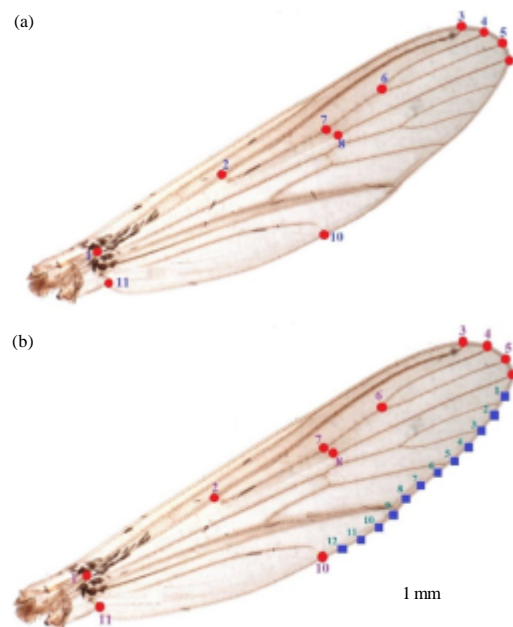


Fig. 1: a) Positions of 11 Landmarks for LM and b) 11 landmarks combined with 12 semi-landmarks for LMSL on *Aedes* wings

Meanwhile discriminant analysis of the canonical create a factor map. Mahalanobis distances were calculated from discriminant analysis to estimate the distance between species. The statistical significance of pairwise Mahalanobis distances between *Aedes* species was computed by non-parametric, permutation tests (1000 cycles) with Bonferroni correction at $p < 0.05$. The accuracy of *Aedes* species classification was based on wing shape and each individual was reclassified by comparing wing similarity to the mean shape of each species based in terms of Mahalanobis distances comparisons (cross-validated classification test). A neighbor-joining tree was constructed based on Procrustes distances to determine the similarity of the shape in each *Aedes* species and added *Culex quinquefasciatus* for a putative outgroup.

LM and LMSL-based approach: For the LM and LMSL-based approaches, there were very similar analytical processes including repeatability test, CS analysis, shape analysis, cross-validated classification, significance test and neighbor-joining tree. However, it has a different element which is the landmark collection. Determining the point of this method is the combination of the 11 Landmarks of LM and the 12 semi-landmarks at the curved line of the vein between landmarks 9 and 10 of the lower angle of the mosquito wing (Fig. 1).

Software: Analyses and computation by both GM approaches in this research made use of specialized software, including CLIC (Collection of Landmarks for Identification and Characterization), package Version 97 (Dujardin, 2011) which is freely available at <https://xyom-clic.eu>. Five modules of CLIC Software were utilized for GM analyses, including COO for digitizing 11 landmarks and 12 semi-landmarks, TET to edit, convert and compute data, MOG to generate centroid size and shape variables, formulate quantile box of size variation and compute Procrustes distances, VAR for analyze size variation and testing statistical difference of size between species and PAD for analysis of shape variation, permutation-based statistical significance of Mahalanobis distances between species and cross-validated reclassifications. Finally, R Software (<https://cran.r-project.org/>) was applied to build neighbor-joining trees for LM and LMSL.

RESULTS AND DISCUSSION

The 45 wings of *Ae. aegypti*, 34 wings of *Ae. albopictus* and 34 wings of *Ae. scutellaris* were used which was the same set for analysis of LM and LMSL.

Before analysis, we repeatedly measured photographs and found that the photograph set used for the analysis had robust quality for pointing landmarks (LM: repeatability size was 0.94 and for shape it was 0.96; LMSM: repeatability of size was 0.92 and shape was 0.94).

Size analyses: CS were used for wing size estimation in both LM and LMSL. Variations of CS are portrayed as quantile boxes for comparison between *Aedes* species in Fig. 2. The CS average indicates that *Ae. aegypti* had the largest wings followed by *Ae. scutellaris* and *Ae. albopictus* in LM while *Ae. aegypti* had the largest wings followed by *Ae. albopictus* and *Ae. scutellaris* in LMSL (Table 1). The statistically significant difference test found that the both LM and LMSL approaches had varying results for CS between species. In LM, *Ae. albopictus* was different from other species while *Ae. scutellaris* was different from other species with LMSL (Table 1).

Shape analysis: After generalized Procrustes analysis, shape variation between species was determined by connecting mean landmark configuration of 11 Landmarks for LM and 11 landmarks combined with 12 semi-landmarks for LMSL by a straight line (Fig. 3). Comparison among the three *Aedes* species has been shown as a factor map for both LM and LMSL as shown in Fig. 4. With the comparison of wing shape between species, we found permutation-based statistically significant differences in Mahalanobis distances for all *Aedes* species with both approaches ($p < 0.05$, Table 2 and 3).

The evaluation of the accuracy of classification of both LM and LMSL (cross-validated classification) showed that LM provided *Aedes* species separation similar to LMSL. The range of cross-validated classification scores based on shape variation in LM was 55-86% while the range for LMSL was 50-86% of which *Ae. aegypti* had the most cross-validated classification scores with both approaches. The similarity of shape for each species of both LM and LMSL was calculated and constructed as shown in the neighbor-joining trees in Fig. 5.

In the research presented here, we evaluated and compared the performance of female *Aedes* mosquito separation, including those from the species *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris*, via LM and LMSL. Analysis of these techniques can be divided into two parts-size and shape. However, with the species identification of mosquitoes, the focus is usually on wing shape, though to investigate the potential of both methods, we had to analyze wing size.

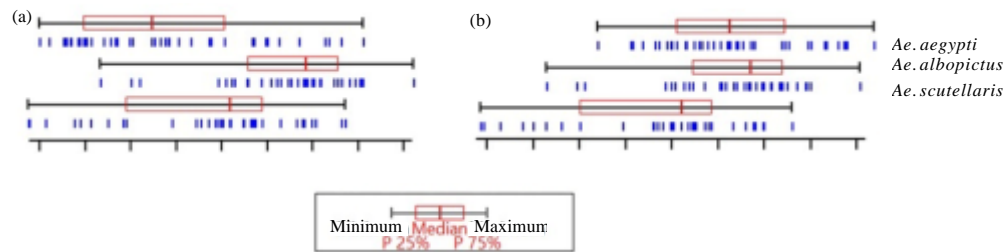


Fig. 2: CS variation of the wings between *Aedes* species (units are mm) shown as quantile boxes: a) SL and b) LMSL). Each box features the median separating the 25 and 75% quartiles

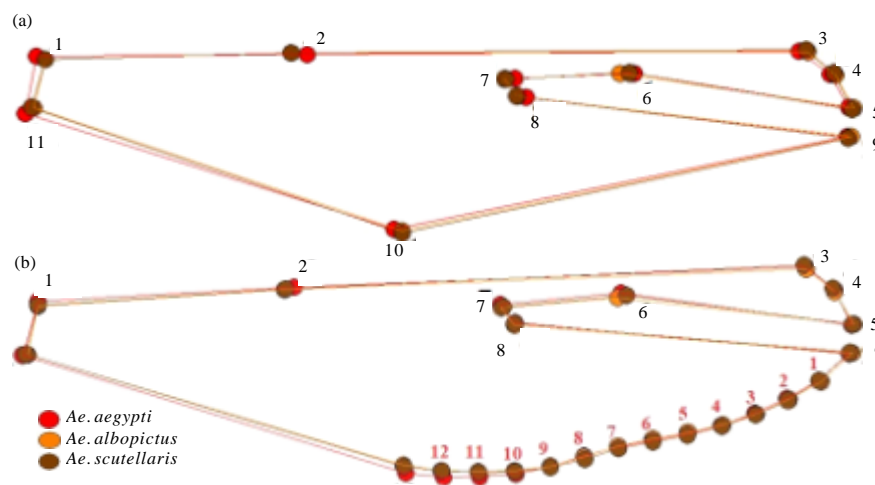


Fig. 3: Superimposition of the mean landmark configurations of *Ae. aegypti* (red), *Ae. albopictus* (orange) and *Ae. scutellaris* (brown): a) LM and b) LMSL

Table 1: Statistical comparisons of wing CS between species in LM and LMSL

Species	Mean±SD of wing CS (mm)	
	LM	LMSL
<i>Ae. aegypti</i>	2.55±0.10 ^a	3.29±0.08 ^a
<i>Ae. albopictus</i>	2.88±0.06 ^b	3.33±0.07 ^a
<i>Ae. scutellaris</i>	2.62±0.09 ^a	3.02±0.12 ^b

^{a, b}Different superscript letters in rows represent statistical differences at $p < 0.05$ (mm: milli meters; mean: average of centroid size; SD: Standard Deviation)

Both LM and LMSL have similar wing size based on CS averaging. However, the permutation-based statistical tests show different CS averages for each species which were different patterns between LM and LMSL, including *Ae. albopictus* that was different from other species in LM while *Ae. scutellaris* was different from other species with LMSL. As such it may be based on the different number of landmark points that affect the results of the size analysis. In addition, varied approaches also affect size

Table 2: Statistical significance of pairwise Mahalanobis distances between species in LM and LMSL

Approaches/species	<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>Ae. scutellaris</i>
LM			
<i>Ae. aegypti</i>	-		
<i>Ae. albopictus</i>	3.25*	-	
<i>Ae. scutellaris</i>	3.28*	1.75*	-
LMSL			
<i>Ae. aegypti</i>	-		
<i>Ae. albopictus</i>	4.59*	-	
<i>Ae. scutellaris</i>	5.03*	3.15*	-

*Statistical differences

analysis. This was consistent with the previous results that compared the findings with trombiculid mites within the genus, *Walchia* in terms of identification of LM and an outline-based GM method that yielded different size patterns between both approaches (Sungvornyothin *et al.*, 2019).

Wing shape is important factor for mosquito species identification using GM. The wing is the organ that

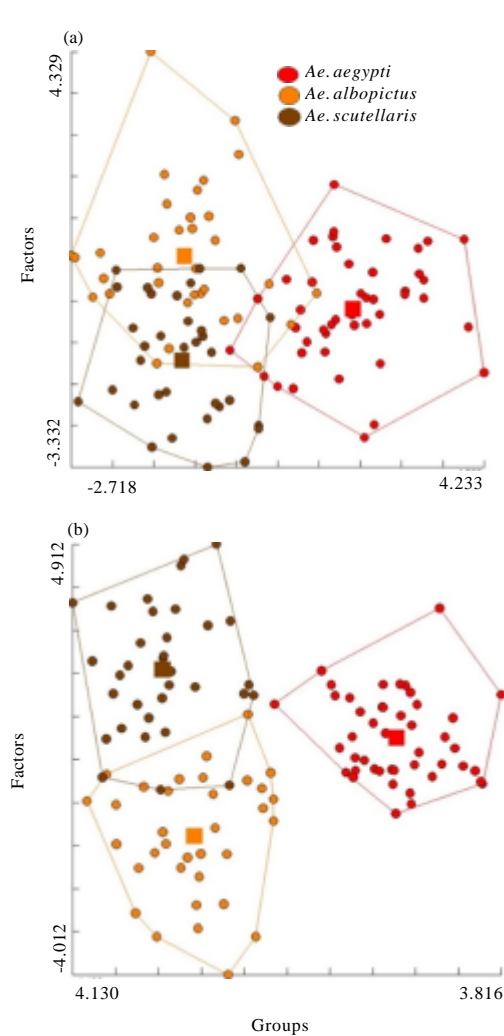


Fig. 4: Factor maps for discriminant analysis resultant from comparison among the three *Aedes* species, including *Ae. aegypti* (red), *Ae. albopictus* (orange) and *Ae. scutellaris* (brown): a) LM and b) LMSL. Each point represented an individual *Aedes* for each group

Species	Accuracy of classification (assigned/observed)	
	LM (%)	LMSL (%)
<i>Ae. aegypti</i>	86(39/45)	86(39/45)
<i>Ae. albopictus</i>	55(19/34)	50(17/34)
<i>Ae. scutellaris</i>	61(21/34)	67(23/34)

displays the most unique identity when exploring intraspecific variations among mosquito populations and differences in the wings are derived from sex (Virginio *et al.*, 2015), geographical location (Demari-Silva *et al.*, 2014), phylogenetic relationships (Sumruayphol *et al.*, 2016), ecological relationships

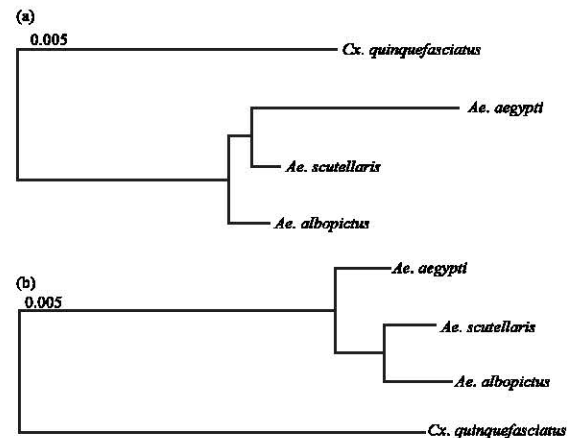


Fig. 5: Neighbor-joining trees based on Procrustes distances between *Aedes* species: a) LM and b) LMSL

(Hidalgo *et al.*, 2015) and types of treatment (Lorenz *et al.*, 2017). While size is a factor that easily changes based on effects of the environment and food received it is unsuitable for species identification (Dujardin, 2011). The results of shape analyses with LMSL indicate the efficacy of *Aedes* identification based on discriminant analysis and permutation-based statistically significant differences in Mahalanobis distances. In addition, comparison of cross-validated classification scores of LMSL with LM as a popular approach yielded that they had similar efficacies for three *Aedes* species separation. However, in certain *Aedes* species such as *Ae. scutellaris*, LMSL can better separate than LM while in other species such as *Ae. albopictus*, LM can better separate than LMSL. In line with earlier research it has been found that LMSL can better identify tsetse fly species than LM. The close relationship of shape between the species expressed as a neighbor-joining tree meant we observed the difference between the approaches caused by 12 semi-landmarks on the LMSL curve.

CONCLUSION

GM is an alternative technique that has become popular for species identification with applications to entomological work. LMSL is one GM technique that has a strong fit for curved positions whereas other GM methods are difficult, like LM. The results of the comparison between LM and LMSL in this study have revealed that they had similar efficacies for three *Aedes* species separation, including *Aedes aegypti*, *Ae. albopictus* and *Ae. scutellaris*, based on shape variation. However, for certain *Aedes* species such as

Ae. scutellaris, LMSL can better separate than LM while for other species, like *Ae. albopictus*, LM can better separate than LMSL.

RECOMMENDATIONS

Therefore, we recommend that before applying this technique, there should be a comparison to other methods to determine the specificity of species of mosquito vector when seeking an appropriate GM technique.

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