

Outline-based geometric morphometric analysis to identify two *Anopheles* and three *Culex* mosquitoes in Thailand

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Abstract. Chaiphongpachara T. 2019. Outline-based geometric morphometric analysis to identify two *Anopheles* and three *Culex* mosquitoes in Thailand. *Biodiversitas* 20: 1866-1872. Geometric morphometric (GM) techniques have become popular for applications in entomology studies, especially mosquitoes. Outline-based methods (OTLs) are one such form of GM technique used to analyze pseudo-landmarks on contours or boundary outlines. This study investigated the efficacy of an OTL to distinguish two species of *Anopheles* mosquitoes including *An. epiroticus* and *An. subpictus* s.l. and three species of *Culex* mosquitoes, including *Cx. quinquefasciatus*, *Cx. visnui* and *Cx. whitmorei* in Thailand and compared outlines, including internal outline 1 (IOL1), internal outline 2 (IOL2) and external outline (EOL) within the mosquito wing to assess the optimal outline for analysis. The results indicated that OTLs were highly effective with certain species and each outline had the potential difference for identification. For size analysis, *An. epiroticus* had a mean perimeter length of IOL2 and EOL length larger than *An. subpictus*. Different sizes between species was found in IOL1, which was statistically significant after Bonferroni test ($p < 0.05$). *Culex* size analysis of IOL2 and EOL demonstrated a statistical difference for all species while size difference patterns between outlines found that IOL1 differed from other outlines. While shapes in two *Anopheles* of all cells were different between species, there was statistical significance based on Mahalanobis distances ($p < 0.05$). Almost all pairwise Mahalanobis distances between *Culex* species of IOL1, IOL2 and EOL established statistical differences, except for pairs of *Cx. visnui* and *Cx. whitmorei* via IOL2 analysis.

Keywords: *Anopheles epiroticus*, *Anopheles subpictus* s.l., *Culex quinquefasciatus*, *Culex visnui*, *Culex whitmorei*, geometric morphometric, outline-based method

INTRODUCTION

At present, geometric morphometric (GM) techniques have become popular based on applications in entomology studies, such as species identification, sexual dimorphism and inspection of morphological variation patterns in each different area (Lorenz et al. 2017; Chaiphongpachara 2018; Chaiphongpachara et al. 2018; Virginio et al. 2015). In recent years, its applications in biological and medical science have grown rapidly (Lorenz et al. 2017). GM is a powerful analytical tool based on the geometry of phenotype, which is commonly used to study insects, especially the family, Culicidae (Wilke et al. 2016; Dujardin 2011). Mosquitoes are one of the target insects for GM as a consequence of their wings, which are bi-dimensional organs suitable for GM analysis (Lorenz et al. 2017; Dujardin 2008). The main reason this technique is applied quite commonly in mosquitoes is that they are medically important insects seeing they act as vectors of human diseases (Lorenz et al. 2017). There is also the problem surrounding their identification according to the gold standard method (morphological identification by taxonomic key) - a number of species of mosquitoes have morphological similarities induced by the difficulty in controlling them.

Malaria is a life-threatening disease and one of the most severe public health problems worldwide, particularly in tropical and subtropical areas (Service 2008). Globally, the

World Health Organization (WHO) reported that there were more than two hundred million malaria cases and more than four hundred thousand deaths in 2016 (World Health Organization 2016). Malaria is caused by the protozoan parasite that belongs to the genus Plasmodium (phylum Apicomplexa) transmitted by female *Anopheles* mosquitoes (Diptera: Culicidae) (Cox 2010). More than 400 formally named *Anopheles* species have been recognized and approximately 80 species of *Anopheles* mosquitoes are regarded as vectors of mosquito-borne diseases (Tainchum et al. 2015). The complexity of the species of *Anopheles* is one of the obstacles in terms of classification. Many *Anopheles* species have very similar morphological characteristics that are difficult to distinguish by morphological methods (World Health Organization 2007). At present, there is an increase in the list of new species of *Anopheles* mosquitoes owing to the development of technology for species identification (World Health Organization 2007).

In Thailand, malaria continues to be a public health problem, especially along the international borders with Cambodia, Myanmar, and Malaysia (Parker et al. 2015). Approximately 74 species of *Anopheles* mosquitoes have been found distributed across different areas according to the specificity of the species in Thailand (Tainchum et al. 2015). Three species of *Anopheles* mosquitoes are recognized as primary malaria vectors in Thailand, including *An. dirus*, *An. minimus*, and *An. maculatus*

(Parker et al. 2015). With this, three species of *Anopheles* have habitats in the forest areas of Thailand along the border area, corresponding to high numbers of malaria cases (Tainchum et al. 2015). *An. epiroticus* is considered to be a potential malaria vector and is widely distributed along the coastal areas of Thailand (Chaiphongpachara and Sumruayphol 2017). Previous research first uncovered evidence in Thailand of *Plasmodium falciparum* and *P. vivax* infection in *An. epiroticus* (Sumruayphol et al. 2010). Meanwhile, *An. subpictus* s.l. is a well-known vector of malaria in Indonesia and Sri Lanka but is not a vector in Thailand (World Health Organization 2007). However, unlike many countries in the Oriental region, *An. subpictus* s.l. is often located in coastal areas, along with the *An. sundaicus* complex, such as *An. epiroticus*, causing difficulty in separating both species.

While, mosquitoes in the genus *Culex* are found more or less worldwide and are nocturnal vectors of many dangerous human diseases, such as Japanese encephalitis (JE) and lymphatic filariasis (LF) (Service 2008; Tolle 2009). According to the World Health Organization, there were approximately 68,000 cases of JE as a viral infection of the human brain and 13,600 to 20,400 deaths globally (World Health Organization 2014). LF (known as elephantiasis) is caused by three species of nematode worms, including *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, that has approximately 120 million infected cases in tropical and subtropical areas of the world (Shenoy 2008; Goel and Goel 2016). Thailand is a country located in tropical areas, and has specifically high species richness of *Culex* mosquito vectors with many outbreaks of *Culex*-borne diseases. As with other genera of mosquitoes, *Culex* mosquitoes have a morphology similar to many other species. Therefore, finding effective alternative techniques to help classify them are important, excepting molecular techniques are powerful tools but also quite expensive.

Outline-based GM method (OTL) is a GM technique used to analyze pseudo-landmarks on contours or boundary outlines (Dujardin et al. 2014; Santillán-Guayasamín et al. 2017). Although OTL has been less popular than landmark-based (LM) GM methods, it has outstanding potential, much more so than LM. Previous research has revealed the efficacy of OTL for *Aedes* mosquitoes identification in Thailand, of which OTL can better separate male *Ae. albopictus* and *Ae. scutellaris* versus LM (Sumruayphol et al. 2016). Recently, studies have demonstrated that an outline-based GM approach is highly effective in identifying certain species of *Anopheles* mosquitoes in Colombia (Jaramillo-O et al. 2015).

Consequently, this study investigated the efficacy of OTL in distinguishing three species of *Culex* mosquitoes, including *Cx. quinquefasciatus* (JE and LF vectors), *Cx. visnui* (JE vector) and *Cx. whitmorei* (LF vector) and two species of *Anopheles* mosquitoes (malaria vectors) including *An. epiroticus* and *An. subpictus* s.l. in Thailand. It also compared external contour and internal outlines within the mosquito wing to assess the optimal outline for analysis with OTL. The results of this research can confirm the effectiveness of OTL for identifying mosquito species with further applications in the field.

MATERIALS AND METHODS

Mosquito collection and identification

Female adults of the *Culex* species were collected using independent mosquito traps (Woodstream Corporation, USA) from Huay Nam Nak village (13°22'36.0"N, 99°16'34.9"E) in Ratchaburi Province, Thailand, at night (6.00 PM- 6.00 AM) during June to August 2015. Three traps were placed about five meters away from houses and inspected once a week. While, two species of *Anopheles* mosquitoes were collected from two areas in Thailand, including *An. epiroticus* from Samut Songkhram Province (13°24'32.52"N 100° 0'41.40"E) and *An. subpictus* s.l. from Ratchaburi Province (13°22'36.0"N, 99°16'34.9"E) using a Mosquito Magnet Independence Mosquito Trap (Woodstream Corporation, USA). Both areas for *Anopheles* collection had distinct environments. Mosquito collecting areas in Samut Songkhram Province are within coastal areas, important habitats for *An. epiroticus* (Chaiphongpachara and Sumruayphol 2017). Although there have been reports surrounding the habitat of *An. subpictus* s.l. in coastal areas, in Thailand, this has not been confirmed clearly, probably owing to the different species of the *An. subpictus* complex. Therefore, we collected *An. subpictus* s.l. in Ratchaburi Province according to previous researchers that found them (Chaiphongpachara T et al. 2018). After collection, all mosquitoes were sent to the laboratory of the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Center for species identification based on morphology using the illustrated keys to the mosquitoes of Thailand (Rattananarithikul et al. 2005a,b).

Wing preparation

Thirty samples per species of *Anopheles* and *Culex* mosquitoes were selected from the random number table. Afterward, the right wing of each female mosquito was removed from the thorax and mounted using Hoyer's medium on a microscope slide with a cover slip (if a wing was damaged, we repeated the sampling). All mosquito wings on slides were photographed under 40x magnification with a digital camera connected to a Nikon SMZ745T stereomicroscope (Nikon Corp., Tokyo, Japan) at a one-mm scale indicated on all photographs.

Outline-based GM method (OTL)

Before OTL analysis, the repeatability index as a measurement error was employed for quality estimation of a pseudo-landmarks plot (Arnqvist and Mårtensson 1998). Three outlines in Figure 1 are depicted, including internal outline 1 (IOL1), internal outline 2 (IOL2) and external outline (EOL), all considered representative in this OTL analysis. For analysis, the wing size of three outlines was assessed by the perimeter of the outlines. Meanwhile, shape variables were computed by the elliptic Fourier analysis based on perimeter coordinates of the outlines. Discriminant analysis permitted the creation of a factor map to explore the degree of wing-shape dissimilarity in each *Culex* and *Anopheles* species.

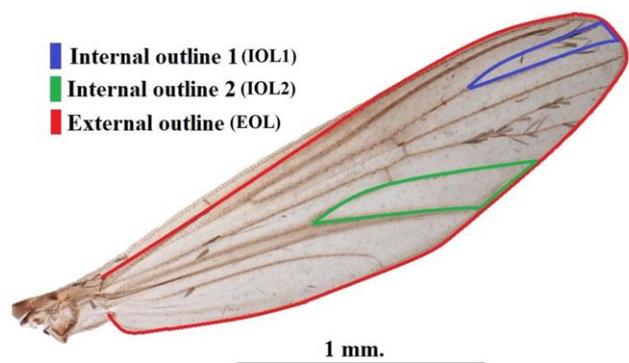


Figure 1. Three outlines for OTL analysis, including IOL1 (blue), IOL 2 (green) and EOL (red)

Further, Mahalanobis distance between species in each outline was computed using the first principal components of the normalized elliptic Fourier coefficients. A non-parametric, permutation-based test (1,000 cycles) with Bonferroni correction was employed for statistically significant difference tests ($p < 0.05$) of the pairwise average perimeter of the outlines and Mahalanobis distance between species. The cross-validated reclassification test based on Mahalanobis distance was utilized to reclassify each individual for monitoring correctly assigned individuals.

Geometric morphometric (GM) software

Five modules of CLIC as GM software (freely available at <https://xyom.io>) were used for outline-based GM analysis in this study. Various modules, including the COO module for outline digitization, the TET module for editing, converting or computing data, the FOG module for processing an outline-based study (including elliptical Fourier analysis, Procrustes superimposition, principal component analyses, discriminant analyses and quantile box of size variation), the VAR module for size analysis, and the PAD module for shape analysis.

RESULTS AND DISCUSSION

The measurement errors of IOL1, IOL2 and EOL for size and outline shape estimation were low (for size < 2%, 5% and 6%, respectively, and < 1%, 4% and 5% for outline shape estimation, respectively).

Anopheles species identification

For size analysis: size variation between *An. epiroticus* and *An. subpictus* s.l. in each cell is portrayed in Figure 1. *An. epiroticus* had a mean perimeter length of IOL2 and EOL larger than *An. subpictus* s.l. (Table 1). Meanwhile, for internal cell 1, *An. subpictus* s.l. had a mean perimeter length that was larger than *An. epiroticus*. Different sizes between *An. epiroticus* and *An. subpictus* s.l. were found in IOL1, which was statistically significant after Bonferroni testing ($p < 0.05$).

For shape analysis: After principal component analysis, which showed there to be shape variations between species in each cell; factor maps from the discriminant analysis were used for evaluating group separation. In Figure 3, factor maps clearly indicate the separation between *An. epiroticus* and *An. subpictus* s.l. in all cells. Meanwhile, shapes of all cells were different between species, which was statistically significant based on Mahalanobis distances (Table 2). Percentages of correct assigning of individuals from cross-validated classification tests have shown a 76-80% range for internal cell 1, 63-70% range for internal cell 2, and 63% range for external cell (Table 3).

Culex species identification

For size analysis: quantile boxes for comparison of mean size variation based on the perimeter of each outline are portrayed in Figure 4. In IOL1, *Cx. whitmorei* had the largest size while *Cx. quinquefasciatus* had the smallest. In IOL2, *Cx. visnui* and *Cx. whitmorei* had the largest size while *Cx. quinquefasciatus* had the smallest. Lastly, *Cx. visnui* had the largest size while *Cx. whitmorei* had the smallest for EOL. The average of the size patterns for each outline was significantly different (Table 4), such as *Cx. whitmorei* differed from other species in IOL1, while in terms of both different outlines, all *Culex* species varied ($p > 0.05$, Table 4).

For shape analysis: the factor maps of the discriminant analysis of wing shape based on each outline indicated *Culex* species separation that was not clearly distinguishable between species (Figure 5).

All pairwise interspecific comparisons of shape between species based on Mahalanobis distance were significant in IOL1 and EOL, while for IOL2, only *Cx. whitmorei* was different from other species ($p < 0.05$, Table 5). For comparisons of the range of cross-validated classification scores between outlines, EOL was greater than IOL1 and IOL2 (63-83% VS 46-63% and 50-60%, respectively, Table 6).

Table 1. Statistical analyses of mean perimeter length in each cell of *Anopheles epiroticus* and *An. subpictus* s.l.

<i>Anopheles</i> species	Mean±Standard deviation (mm)		
	IOL1	IOL2	EOL
<i>An. epiroticus</i>	1.59±0.02 ^a	2.52±0.03 ^a	6.16±0.14 ^a
<i>An. subpictus</i> s.l.	1.69±0.02 ^b	2.48±0.03 ^a	6.04±0.15 ^a

Note: In each row, different superscript letters indicate statistical differences at $p < 0.05$

Table 2. Comparisons of shape differences between *Anopheles epiroticus* and *An. subpictus* s.l. based on Mahalanobis distances in each cell

Cells	Mahalanobis distances scores between species	p-values
IOL1	2.50	<0.001**
IOL2	2.17	0.021*
EOL	2.92	0.002**

Note: * indicates significant differences at $p < 0.05$ and ** indicates significant differences at $p < 0.01$

Table 3. Cross-validated classification of *Anopheles epiroticus* and *An. subpictus* s.l. based on shape in each cell

<i>Anopheles</i> species	Percentages of correct assigning of individuals (assigned/observed)		
	IOL1	IOL2	EOL
<i>An. epiroticus</i>	76 (23/30)	63 (19/30)	63 (19/30)
<i>An. subpictus</i> s.l.	80 (24/30)	70 (21/30)	63 (19/30)

Table 4. Statistical analyses of mean perimeter length in each cell of *Culex quinquefasciatus*, *Cx. visnui* and *Cx. whitmorei*

<i>Culex</i> species	Mean values of size and standard deviation (mm)		
	IOL1	IOL2	EOL
<i>Cx. quinquefasciatus</i>	1.70±0.03 ^a	2.37±0.06 ^a	6.22-0.53 ^a
<i>Cx. visnui</i>	1.78±0.02 ^a	2.45±0.05 ^b	6.29-0.27 ^b
<i>Cx. whitmorei</i>	1.82±0.01 ^b	2.45±0.01 ^c	6.10-0.90 ^c

In each row, different superscript letters indicate statistical differences at $p < 0.05$.

Table 5. Statistical differences in Mahalanobis distance between *Culex* species in each outline

Out-lines	Species	<i>Cx. quinquefasciatus</i>	<i>Cx. visnui</i>	<i>Cx. whitmorei</i>
IOL1	<i>Cx. quinquefasciatus</i>	-		
	<i>Cx. visnui</i>	1.88*	-	
	<i>Cx. whitmorei</i>	2.02*	2.07*	-
IOL2	<i>Cx. quinquefasciatus</i>	-		
	<i>Cx. visnui</i>	2.83*	-	
	<i>Cx. whitmorei</i>	2.77*	1.92	-
EOL	<i>Cx. quinquefasciatus</i>	-		
	<i>Cx. visnui</i>	2.81*	-	
	<i>Cx. whitmorei</i>	3.57*	3.85*	-

Note: * = Statistical differences

Table 6. Cross-validated classification scores of three species of *Culex* mosquitoes in each outline

<i>Culex</i> species	Percent correctly assigned individuals (assigned/observed)		
	IOL1	IOL2	EOL
<i>Cx. quinquefasciatus</i>	46 (14/30)	50 (15/30)	63 (19/30)
<i>Cx. visnui</i>	50 (15/30)	56 (17/30)	70 (21/30)
<i>Cx. whitmorei</i>	63 (19/30)	60 (18/30)	83 (25/30)

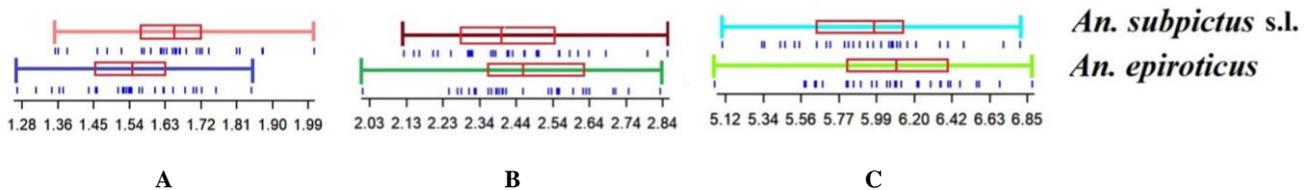


Figure 2. Comparisons of size variation (in mm) between species *Anopheles* in each cell, including: A. IOL1, B. IOL2, C. EOL. Each quantile box shows the group median that separates the 25th and 75th quartiles. Vertical bars under the boxes represent the wing sizes of individual mosquitoes.

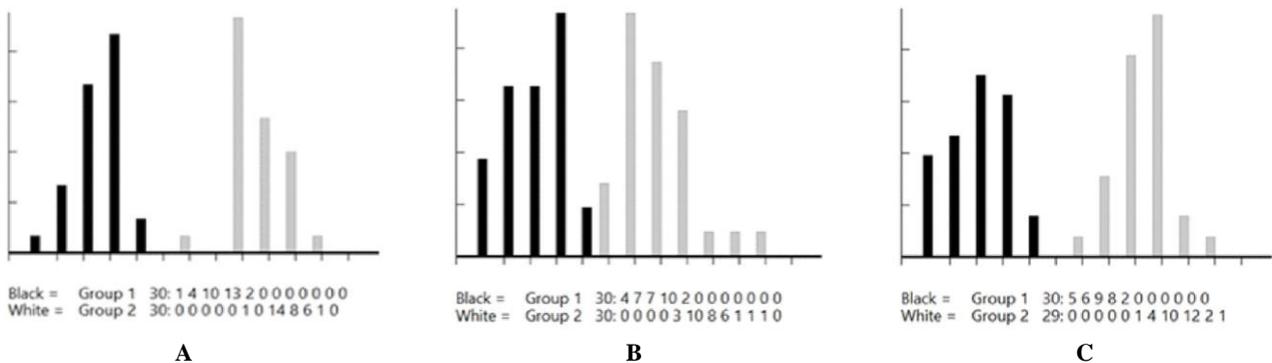


Figure 3. Factor maps of the two discriminant factors derived from shape variables for *Anopheles epiroticus* (black) and *An. subpictus* s.l. (grey) in each cell, including: A. IOL1, B. IOL2, C. EOL

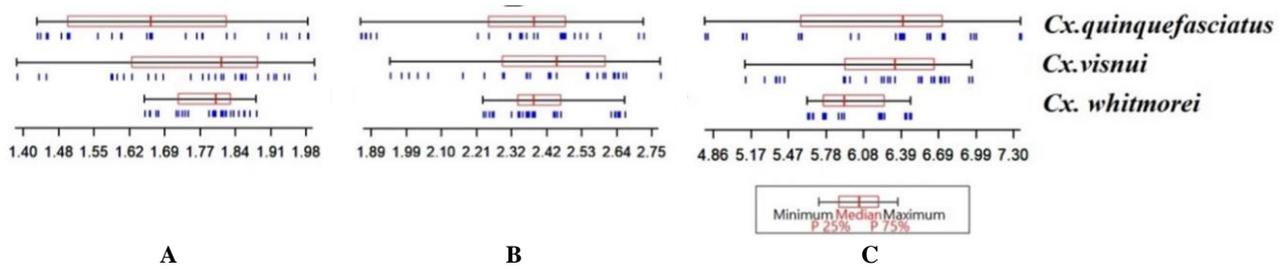


Figure 4. Wing perimeter variation between *Culex* species (mm) from OTL analysis with IOL1 (A), IOL2 (B) and EOL (C). Each quantile box features the group median expressed as median with the 25th and 75th quartiles. Vertical bars under the boxes represent the perimeter sizes of the individual sample

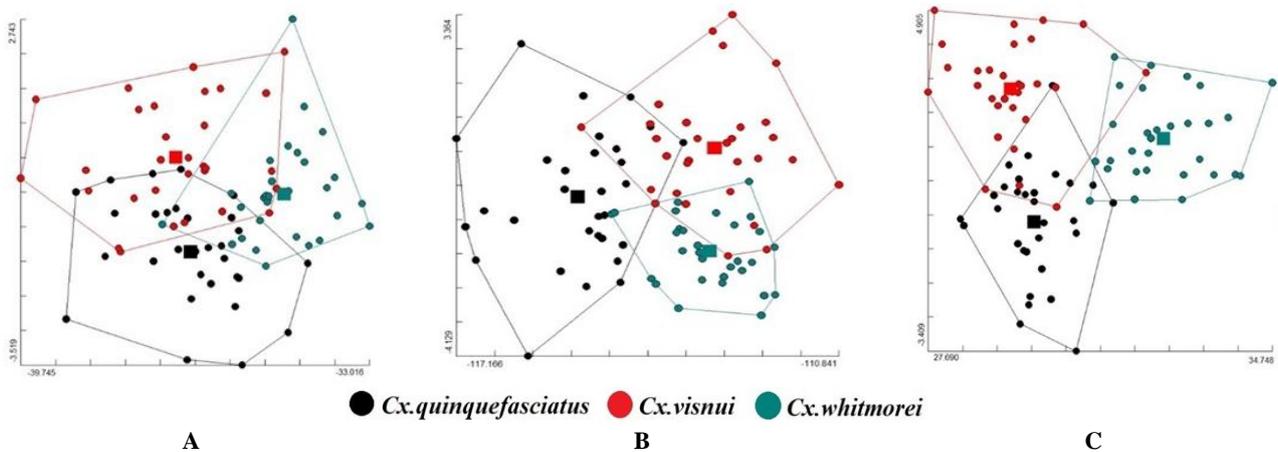


Figure 5. Factor maps of the two discriminant factors (DFs; horizontal axis is the first DF, vertical axis is the second DF) derived from shape variables of the *Culex* species, of which each point represents an individual (A: IOL1, B: IOL2, C: EOL)

Discussion

This research tested the efficiency of discrimination between three *Culex* species, including *Cx. quinquefasciatus*, *Cx. visnui*, and *Cx. whitmorei*, and two *Anopheles* species including *An. epiroticus* and *An. subpictus* s.l. in Thailand using an outline-based GM approach. The results of the study revealed the potential for discrimination of *Culex* and *Anopheles* mosquitoes but there was a difference between each cell for outline-based GM analysis.

Anopheles epiroticus and *An. subpictus* s.l.

Through size analysis, differences between *Anopheles* species were found in IOL1, while size differences were not found in IOL2 and EOL. The difference of shape between mosquitoes species in each cell may emanates from the specificity between species of mosquitoes with internal and external cells of wings. Previous research has compared two GM methods, including landmark-based and outline-based GM approaches for identification of mosquito vectors in Ratchaburi Province, Thailand, and the results of size analysis revealed that both methods are different (Chaiphongpachara 2018). This clearly indicates

that even though it is the same wing, the different positions of the analysis can affect the results of the size between species. However, the size factor is not more important than the shape factor for species identification (Dujardin 2008; Dujardin 2011). In general, the size of adult mosquitoes is often the result of the influence of the environment on the habitat of the larval stage, such as temperature and food quality (Lorenz et al. 2017).

The shape of the wings is popularly used in GM analysis to establish species of mosquito vectors because they are expressed from genetics that is specific to the species (Louise et al. 2015; Lorenz et al. 2017). Shape analysis has demonstrated differences between species in all cells, reflecting the effectiveness of the method for discrimination between both mosquitoes. In accordance with previous research, an outline-based GM approach has proven effective in species identification of many arthropods, such as mosquitoes (Sumruayphol et al. 2016), trombiculid mites within the genus *Walchia* (Sungvornyothin et al. 2018), stomoxys flies (Changbunjong et al. 2016), and tsetse flies (Kaba et al. 2017). These results suggest that each outline on the same wing of a mosquito has the power to discriminate each

species of mosquitoes differently. Cross-validated classification scores revealed that IOL1 is most effective for discrimination between *An. epiroticus* and *An. subpictus* s.l., followed by IOL2 and EOL, respectively. This corresponds to the results of size analysis, which indicates that IOL1 exhibits statistical differences in size

Culex quinquefasciatus, *Cx. visnui*, and *Cx. whitmorei*

Size analysis of IOL2 and EOL exhibited statistical differences for all species while size difference patterns between outlines determined that IOL1 differed from other outlines. In addition, the mean perimeter between *Culex* spp. was different for each outline. This revealed that the outline position affected the results from the size analysis, which may be different when using varied outlines for OTL analysis, even in the same set. Certain outlines are not strong representatives for definition when estimating the total size of mosquito wings. However, size is not a factor for species classification owing to variation occurring readily (De Carvalho et al. 2017; Dujardin 2008; Hidalgo et al. 2015).

Overall, shape analysis of OTL uncovered the effects of the three *Culex* species. Almost all pairwise Mahalanobis distances between species of IOL1, IOL2, and EOL established statistical differences based on non-parametric permutation tests, except for the pair of *Cx. visnui* and *Cx. whitmorei* with the IOL2 analysis. This research was consistent with previous studies conducted in terms of species identification of *Aedes*, *Culex*, and *Anopheles* mosquitoes in Thailand, which found that OTL was a potential technique (Chaiphongpachara 2018). Comparison of the efficiency of each outline for species separation via cross-validated classification scores established that EOL had the highest potential, especially for *Cx. whitmorei* as a LF vector. This corresponds to previous research where the application of EOL and IOL for testing separation of 11 *Anopheles* species in Colombia determined that both outlines had different results (Jaramillo-O et al. 2015).

In conclusion, an outline-based GM approach is one of the reliable GM approaches for discriminating mosquito species based on the different analysis of contours or an internal cell of the wing between species. This research has shown the efficiency of discrimination between three *Culex* and two *Anopheles* mosquitoes in Thailand using a GM approach. In addition, the results also indicate that IOL1 is the most suitable position for discriminating both species of *Anopheles* mosquitoes. While, EOL is more effective for species separation than IOL across all three *Culex* species. The results of this study are a guide to the use of an outline-based GM approach to solve the morphological similarities between species of mosquito further.

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