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Geometric morphometrics approach towards discrimination of three member species of *Maculatus* group in Thailand

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Abstract

Members of the *Maculatus* group are important malaria vectors in the border regions of Thailand. However, the role of each species in malaria transmission remains unclear because of their highly similar morphologies, making them difficult to be differentiated. Whereas *An. pseudowillmori* may be identified by the color pattern of some scales on abdomen and wings, the distinction between *An. maculatus* and *An. sawadwongporni* relies on the wings only. Scales are labile structures, as they may be accidentally removed during capture and transportation to the laboratory. To discriminate among the species of this complex, we tested the suitability of geometric techniques. Shape variables were used as input for discriminant analyses and validated reclassification. Both landmark- and outline-based geometric techniques disclosed significant differences between the three species. For the delicate *An. maculatus* - *An. sawadwongporni* distinction, the outline-based approach appeared as the most promising, with validated reclassification scores reaching 93%, as compared to 77% obtained by landmark data. For *An. pseudowillmori*, reclassification scores were 100% and 94%, respectively. Geometric morphometrics may provide an alternative and useful complement for discriminating members of the *Maculatus* group.

Graphical Abstract

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Keywords

Geometric morphometrics; species identification; *Anopheles maculatus*; *Anopheles sawadwongporni*; *Anopheles pseudowillmori*

1. Introduction

Malaria, a vector-borne disease, is a significant public health issue in tropical and subtropical regions, with an estimated 219 million cases having occurred globally in 2017 (World Health Organization, 2017). Although there has been a decrease in the number of malaria cases in Thailand, it remains one of the most important health issues here, with 2,969 cases being reported in 2017 (Bureau of Epidemiology, 2017). Malaria is particularly prevalent in border regions of Thailand (Parker et al., 2015), with the high incidence of 42.78 cases per 1,000 people reported in Tak Province (Bureau of Epidemiology, 2017). Thus, effective management is required to control malaria transmission in this area.

Female mosquitoes of the genus *Anopheles* transmit human malaria parasites. Therefore, mosquito control is an important strategy for managing malaria epidemics. Successful control requires information on the biology, ecology, behavior, and genetics of *Anopheles* mosquitoes (Sriwichai et al., 2016). Because the biology, behavior, and epidemiological characteristics vary between species, it is imperative to know which species occur in the area of interest in order to plan and monitor vector control (Tainchum et al., 2015).

There are currently 525 species, 475 formally names ones and more than 50 unnamed ones of *Anopheles* in the world (Harbach, 2018), and approximately 70 of which are important vectors of malaria with about 40 regarded as the dominant malaria (Harbach, 2018; Hay et al., 2010; Minkeu and Vernick, 2018). In Thailand, 73 species of *Anopheles* mosquitoes

have been reported, among which three groups (Dirus group, Minimus group, and Maculatus group) have been recognized as the main malaria vectors (Tainchum et al., 2014). These species complexes consist of several closely related, morphologically indistinguishable species that do not interbreed with the other species in the group (World Health Organization, 2007).

The Maculatus group (also called *Anopheles maculatus* complex) in the subgenus *Cellia* has nine sibling as a super complex and are variously distributed in many areas from India to Indonesia and the Philippines (Manguin et al., 2008). Seven species from the Maculatus group have been found in Thailand, including *An. sawadwongporni*, *An. maculatus* s.s., *An. dravidicus*, *An. notanandai*, *An. willmori*, *An. pseudowillmori*, and *An. rampae* (Parker et al., 2015). Although members of this species complex are known to be important malaria vectors in border regions of Thailand, their roles in malaria transmission remain unclear because of difficulties in identifying them in the field using morphological keys (Singh et al., 2012; Dujardin, 2008). The key characters to separate such morphologically close species of mosquitoes are labile characters such as scales on the wings, and other taxonomic characters could be damaged or lost because of collection, time and conservation technique (Sumruayphol et al., 2016). For example, three species that are common in Thailand including *An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori*, according to the illustrated keys to the mosquitoes of Thailand (Rattanaarithikul et al., 2006), between *An. maculatus* and *An. sawadwongporni*, the final distinction depends on a tuft of scales on the vein bifurcation of radius 2 and radius 3 (Fig. 2). Black scales are found in *An. sawadwongporni* but not found in *An. maculatus*, the scales of which are of pale color. While *An. pseudowillmori* may be identified by the color pattern of some scales on both abdomen and wings. These characters were obvious when scales are present, however many specimens did lost these scales after their collection. In this situation, morphological identification could be helped by techniques using other kind of characters, such as genetic traits or geometric features. Genetic techniques such as DNA barcoding are relatively expensive and require specialized training, and their use could be restricted by operational problems (Ruangsittichai et al., 2011). In contrast, geometric techniques are fast and low-cost methods, and have been frequently shown to be a significant help for morphological identification (Dujardin, 2008).

Geometric morphometric (GM) analysis is a modern technique (“modern” in opposition to the traditional morphometric practices) for analyzing variation in shape and size (Rohlf and Marcus, 1993). Two general methods are applied, the landmark-based and the outline-based methods (Dujardin et al., 2014). Landmark-based approach aim at analyzing size and shape based on the relative position of anatomical landmarks (also called true landmarks) (Rohlf, 1990). While, outline-based approach evaluated the size and shape based on pseudo-landmarks describing contours or boundary outlines (Lestrel, 1989). These techniques are inexpensive, fast, and require few equipment (Garros and Dujardin, 2013; Lorenz et al., 2017; Dujardin, 2017). Previous studies showed that these techniques can successfully distinguish different species of mosquitoes, such as *Anopheles* and *Aedes* (Dujardin, 2008; Sumruayphol et al., 2016; Jaramillo-O et al., 2015; Dujardin et al., 2014; Motoki et al., 2012; Lorenz et al., 2012; Chaiphongpachara, 2018). The landmark-based technique is the main approach on wing venation patterns, but it has been shown that the outline-based

technique could be relevant too (Dujardin et al., 2014). Needless to say, the outline-based technique can become the only relevant approach for organs lacking true landmarks (Faille et al., 2007; Tatsuta et al., 2011; Boussès et al., 2013), for example, eggs (Santillán-Guayasamín et al., 2017). However, not all species can be satisfactorily classified by the outline-based technique, making it necessary to examine its feasibility in each situation (Ruangsittichai et al., 2011).

With the idea to select the best helping strategy to improve morphological species identification, we examined whether landmark- and outline-based techniques could be used to discriminate between three members of the *Maculatus* group including *An. metadatas*, *An. sciawdwongporni* and *An. pseudowillmori* in Tak Province, Thailand.

2. Material and methods

2.1 Study sites and mosquito collection

Adult *An. maculatus*, *An. sciawdwongporni* and *An. pseudowillmori* were collected from January to December 2015 in four villages (Suan Oi, Komonae, Nong Bua, and Tala Oka) of Tha Song Yang District, which had the highest prevalence of malaria in Tak Province, northwestern Thailand (Sriwichai et al., 2016) (Fig. 1). The study region is surrounded by mountains and hilly forests and comprised rice fields, rivers or streams, and cassava plantations along the side of the road, all of which are potential breeding sites for *Anopheles*. CDC-light traps (LT) (BiQuip model 2836BQ, with a 6-volt battery, USA) were placed overnight at 30 selected houses in each village, which had previously reported malaria cases. Mosquito collection was performed monthly for five consecutive nights from 18:00 to 06:00 (12 h) by hanging CDC-LT at 20 m away from houses. Mosquitoes were transferred from the trap into 60 ml specimen containers in early morning each day and transported alive to a laboratory at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok. They were then transferred to a -20°C freezer until morphological identification.

2.2 Morphological identification of the reference materials

In our study, the accuracy of morphometric identification was compared to that of the morphological identification. We took a special care of using only unambiguous specimens for morphological identification. Out of 543 individuals examined, only 243 were retained because the key characters were clearly visible. We thus retained for our analyses unambiguously identified mosquitoes: 200 *An. metadatas*, 43 *An. sciawdwongporni* and 36 *An. pseudowillmori* (Table 1). All of them could be submitted to the outline analysis (sample sizes were 200, 43 and 36), whereas for the anatomical landmark digitization, some specimens were discarded (sample sizes were 191, 42 and 36, see Table 1). We discarded morphologically dubious specimens to create a reliable scale, a reference data on which to evaluate the GM diagnostic. For the classification tree, we included 30 *Culex quinquefasciatus* as a putative outgroup.

2.3 Geometric morphometric analyses

2.3.1 Preparation of mosquito wings—The right wing of each mosquito specimen was detached from thorax, removed wing scales using needle carefully for clear vision on the wing vein and mounted on a glass microscope slide in Hoyer's solution. To avoid image distortion, wings were placed at the center of the microscope field. Landmarks and outlines were digitized on pictures taken with a Nikon DS-Ri1 SIGHT digital camera connected to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) under 40 x magnification. A scale bar (1 mm. in length) was visible on every picture.

2.3.2 Landmark-based analysis—In the landmark-based analysis, 17 wing landmarks were selected (Fig. 3a). Wing size was measured as the centroid size (CS), i.e., the square root of the sum of squared distances of the 17 landmarks from their centroid (Bookstein, 1991). Wing shape was computed as orthogonal projections of aligned specimens on the Euclidean plane tangent to the consensus form (generalized Procrustes analysis, Rohlf and Slice, 1990).

2.3.3 Outline-based analysis—The contour of the fourth posterior cell was selected (Fig. 3b) after comparison of reclassification scores derived from other options (details not shown). The length of the perimeter of this contour (PER) was used as an index of wing size, and an elliptic Fourier analysis (EFA) (Kuhl and Giardina, 1982) was used to construct the shape variables, i.e., the normalized elliptic Fourier coefficients (NEF).

2.3.4 Contribution of size to species variation—Allometry is the relationship between size and shape that affects each other. Size and shape are not completely independent attributes. The change of shape due to size variation is called an “allometric” effect. The possible allometric effect in shape-based distinction of species was evaluated by the linear correlation coefficient between global size and the first discriminant factor derived from shape variation (Dujardin, 2008).

2.3.5 Statistical analyses—The measurement error of both methods was evaluated using the repeatability (R) index (Arnqvist and Mårtensson, 1998). Twenty wings per species selected randomly were tested, digitized twice by the same person (Dujardin et al., 2010).

Wing size variation between species derived from both techniques was illustrated by quantile boxes (Fig. 4). Statistical significance of mean values of wing CS or PER between species was examined using non-parametric permutation tests (1,000 runs) with Bonferroni correction at P -values < 0.05 .

In both approaches, wing shape variation between species was illustrated by the superposition of the mean forms (Figs. 5 and 6). The shape divergence for each species was measured by a discriminant analysis (DA) and illustrated by a factor map (Fig. 7). The corresponding Mahalanobis distance were tested by non-parametric tests (permutations, 1000 runs), with Bonferroni correction as significance at P -values < 0.05 .

For assessing how the classification results could be generalized to an independent data set, each individual was removed iteratively and its assignment computed after the analysis of the remaining data (“leave-one-out” method, also called “validated” reclassification) (Manly, 2004).

To illustrate the patterns of morphological divergence among species, a classification tree was computed (Fig. 8). Its stability was tested by the bootstrap technique on input data. The method is explained in Couette et al. (2005) and has been used on *Aedes* mosquitoes by Morales-Vargas et al. (2013). It consists in the generation of numerous (here 10000) data sets of the same size as the initial one by random resampling with replacement of the variables. Each random data set is then analyzed by using the same dissimilarity index and tree-reconstruction method (here single-linkage hierarchical classification algorithm). Finally, the frequencies of each observed cluster in the bootstrapped trees are estimates of their confidence intervals.

2.3.6 Software—Two different software packages were used to repeat and compare results, the CLIC package version 97 (Dujardin and Slice, 2007) (<https://xyom-clic.eu>) and the recent online morphometric package, XYOM (<https://xyom.io>). The topology of the single-linkage hierarchical classification tree as built by XYOM was compared to the UPGMA tree as computed by the R software (<https://cran.r-project.org/>).

3. Results

3.1 Repeatability

The precision of both GM techniques between digitization sets of repeated measurements for the same images showed high scores for both size and shape. The repeatability of the centroid size was as high as 0.97, and slightly higher for the perimeter (0.99). For shape, the repeatability was 0.92 for both landmark-based and outline-based shape data.

3.2 Wing size variation

The global size variation as derived from the landmark- and outline-based analyses disclosed similar patterns for the three species. Significant size differences were disclosed between them ($P < 0.05$; Table 2 and Fig. 4), with *An. pseudowillmori* having the largest average size (CS = 2.79 mm; PER = 2.63 mm) and *An. sawadwongporni* having the smallest one (CS = 2.64 mm; PER = 2.41 mm).

3.3 The effect of size variation on discrimination by shape

Allometric residue of the first discriminant factor derived from landmark-based analysis was 0%, which means that size differences did not influence shape divergence between species. On the contrary, a 34% effect of size variation on shape was observed for the outline-based method.

3.4 Wing shape variation

When superposing the mean wing shapes of *An. maculatus*, *An. sawadwongporni*, and *An. pseudowillmori*, the shape of *An. pseudowillmori* appeared to be the most distinct one (Fig.

5). The examination of the superposed fourth posterior cell contour of the three species also suggested that *An. pseudowillmori* was clearly distinct from the other two species (Fig. 6).

In the graphical output of the landmark-based discriminant analysis, more overlapping could be observed than in the outline-based analysis (Fig. 7). Despite some overlap, wing shape was significantly different among all three species (pairwise Mahalanobis distances, 1,000 runs: $P < 0.05$). The outline data allowed us to discriminate *An. pseudowillmori* without any overlapping (Fig. 7b). While *An. maculatus* and *An. sawadwongporni*, although found overlapping between groups, half of individuals in both groups were separated especially in outline-based discriminant analysis.

Graphical outputs suggested a consistently better discrimination power of the outline approach (Fig 7). This was confirmed by the validated reclassification: higher correct assignation scores were obtained when performed on the outline than on the landmark data (95% vs 81%, respectively, see Table 3). In both approaches, *An. pseudowillmori* was distinguished from the other species with high confidence (100% and 94%). While *An. maculatus* and *An. sawadwongporni* still have high assignment scores (93%) in outline-based method. *Culex* sp., as expected for another genus, was never mixed with the *Anopheles* group, and this complete separation was confirmed by the bootstrap performed on the single linkage hierarchical classification trees: after 10000 bootstraps, no other topology was generated than the one shown in Fig. 8. Thus, *An. pseudowillmori* was always distinct from the other two species, with *An. maculatus* and *An. sawadwongporni* lying closer to each other.

4. Discussion

In this study, we intentionally used non-damaged specimens to have a clear morphological reference to the GM diagnostic. The use of non-damaged wings was our guarantee to correctly evaluate the diagnostic power of GM. In our study, 200 *An. maculatus*, 43 *An. sawadwongporni* and 36 *An. pseudowillmori* specimens were used for GM analysis, which is the number that is greater than the minimum number of samples per species that must be analyzed for an accurate differentiation of the species using GM. the number of specimens should be twice the number of landmarks. However, the use of a limited set of first principal components (thus capturing less shape information) may represent a statistical solution when this condition is not met. For the outlines which may be represented by a very high number of coefficients (each harmonic contains 4 coefficients, and frequently at least 20 harmonics are necessary), the principal component ploy is always used.

4.1 Wing size variation, and its effect on discrimination by shape

Both landmark- and outline-based analyses revealed significant differences in wing size among the three species examined. Many factors, such as temperature, humidity, density and food quality or quantity, can affect the size (Jirakanjanakit et al., 2007; Morales-Vargas et al., 2010; Kuclu et al., 2011; Gómez, 2014; Hidalgo et al., 2015), but the between-species size variation is likely to have also an evolutionary significance (Dujardin, 2008). Therefore, its possible contribution to species shape-based separation does not make problem in our study. Shape as computed by the geometric approach is free of the isometric change of size,

it is not necessarily completely free from the allometric change. This contribution of allometry is frequent in case of striking divergence in size, which in our study was not apparent (see the figure on quantiles), meaning that we compared species on pure shape. Species distinction was based exclusively on shape variables not on size estimations. In this study, size is not used to identify species. Size variation was shown as a complementary information.

4.2 Wing shape variation

Wing venation geometry is apparently similar among different mosquito genera and species, but subtle changes in the relative position of some veins may be detected, making it an important characteristic for geometric analyses (Garros and Dujardin, 2013). Dujardin et al. (2014) showed that the outline-based method could distinguish between *An. strodei* and *An. oswaldoi* by axillary cell, while Sumruayphol et al. (2016) successfully used the external contour to identify three closely related species of *Aedes*. The ability of the outline-based method for species identification can vary with the internal wing cell selected for discrimination (Francoy et al., 2012). We selected the contour of the fourth posterior cell after testing two other, less performing options (details not shown). We found that both the landmark-based and outline-based methods could quantify the wing shape differences between the species examined here with relatively high accuracy, with significant differences being found among all three species. Furthermore, both the landmark configurations and the contour shapes of the fourth posterior cell provided similar tree topologies.

Even if *Culex* spp. was added as a putative outgroup, the classification trees did not assume to be an estimate of phylogenetic relationships, they showed the morphological similarity pattern among two genera and three species of the Maculatus group. In the tree topologies derived from both methods describing shape differences, *An. maculatus* was close to *An. sawadwongporni*, while *An. pseudowillmori* remained clearly separated from the two others (Fig. 8). The species segregation pattern of *An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori* based on wing shape was consistent with the taxonomy of *Anopheles* species members in the Maculatus group (Rattananarithikul et al., 2006). The wing shape was also in agreement with phylogenetic studies on members of the Maculatus group based on the second internal transcribed spacer (ITS2), indicating that *An. pseudowillmori* was distant from other species, while *An. sawadwongporni* and *An. maculatus* were similar (Ma et al., 2006; Singh et al., 2012). These results add confidence to the use of GM as complementary tool helping the morphologist to discriminate species of the Maculatus group.

For the landmark-based analysis, we used a few landmarks dispersed on wing at the intersection between veins, while for the outline-based analysis we used the contour of the fourth posterior cell. In addition to the capture of shape in different areas of the wing, there was also a difference in the statistical processes to compute size and shape: either Procrustes superimposition for the landmarks or Elliptic Fourier analysis for the outlines. These differences could hardly produce the same allometric patterns.

Both morphometric approaches provided comparable performance for the recognition of *An. pseudowillmori*, which is thought to be a potential malaria vector in Thailand, particularly in the northern region (Walton et al., 2007), but they were of unequal importance in separating

the two closest species, *An. maculatus* and *An. sawadwongporni*. *An. maculatus*, which is considered a primary malaria vector in southern Thailand and peninsular Malaysia (Walton et al., 2007), could not be completely distinguished from *An. sawadwongporni* when using landmark data (179 correctly assigned wings out of 233, i.e., 77%). Using outline data however, it was possible to separate these two sibling species with relatively high accuracy (227 correctly assigned wings out of 243, i.e., 93%).

5. Conclusions

Our results showed that modern morphometric techniques could represent a valuable help and sometimes a possible alternative for specimens ambiguously defined. They can be used in addition to the classical morphological identification when diagnostic characters are accidentally lost. Because modern morphometrics is a non-traumatic or poorly traumatic technique, it does not prevent further use of molecular techniques, if required. In this study, the discrimination of *An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori* which are highly similar morphologies by GM is one evidence of how to solve taxonomic difficulties. GM found to be highly effective especially in outline-based method for *An. pseudowillmori* segregation.

The advantages of the morphometric approach are worth noting: it is fast, inexpensive, not needing any new equipment other than the standard one used in a laboratory of entomology, and not requiring highly specialized skills in the group of organisms under study. Thus, modern morphometries and related classification techniques hold great promise for improving the identification and control of malaria vectors in the future, which will allow more appropriate planning for vector control.

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Highlights

- Members of *Anopheles maculatus* complex were characterized by landmark-based and outline-based geometric morphometries.
- Outline-based method was the most appropriate technique for distinguishing between *An. maculatus* and *An. sawadwongporni*.
- In both approaches, *An. pseudowillmori* was separated from the other species with high confidence.

Fig. 1.
Location of the study sites in Tha Song Yang District, Tak Province, Thailand.

Fig. 2.

Top: wing of *Anopheles maculatus*, bottom: wing of *An. sawadwongporni*. These two species are morphologically distinguished at the last steps of the dichotomic key, thanks to the color of the scales at the bifurcation of the R2+3 vein (Rattarithikul et al., 2006, pages 75-77). For *An. sawadwongporni* it is dark, while it is pale for *An. maculatus*. In case of damaged specimens, these scales may be lost (it was the case of more than 50% of our collected materials) and the morphological identification is compromised.

Fig. 3.
Position of (a) the 17 landmarks and (b) the contour of an internal cell, the fourth posterior cell, on *Anopheles* wings.

Fig. 4. Variation in the wing CS of *Anopheles maculatus*, *An. sawadwongporni*, and *An. pseudowillmori*. Values represent (a) the mean CS derived from the landmark-based method and (b) the mean perimeter length derived from the outline-based method. Dots represent each individual. Each quantile box plot shows the group median that lies between the 25th and 75th quartiles.

Fig. 5.
Superposition of the mean landmark configurations of *Anopheles maculatus*, *An. sawadwongporni*, and *An. pseudowillmori*.

Fig. 6.
Superposition of the outlines of *Anopheles maculatus*, *An. sawadwongporni*, and *An. pseudowillmori*.

Fig. 7.
Factor maps derived from landmark-based (a) and outline-based (b) geometric morphometric discriminant analyses.

Fig. 8. Single linkage hierarchical classification trees based on the Mahalanobis distances of (a) landmark-based and (b) outline-based analyses. *Culex quinquefasciatus* was used as a putative outgroup.

Table 1:

Numbers of individuals that were included in the geometric morphometric analyses.

Method/Species	<i>An. maculatus</i>	<i>An. sawadwongporni</i>	<i>An. pseudowillmori</i>	Total
Landmark-based	191	42	36	269
Outline-based	200	43	36	279

Table 2:

Mean wing CS and mean perimeter length of species in the *Maculatus* group.

Species	Landmark-based method		Outline-based method	
	Mean (min–max) (mm)	S.D.	Mean (min–max) (mm)	S.D.
<i>An. maculatus</i>	2.68 (2.21–3.07) ^a	0.15	2.51 (2.07–3.04) ^a	0.16
<i>An. sawadwongporni</i>	2.64 (2.11–2.99) ^a	0.15	2.41 (2.15–2.69) ^a	0.13
<i>An. pseudowillmori</i>	2.79 (2.20–3.14) ^b	0.22	2.63 (2.20–2.91) ^b	0.18

Different superscript letters indicate significant differences between species at $P < 0.05$. Mean: average CS and perimeter length calculated using the landmark-based and outline-based methods, respectively; min: minimum; max: maximum; S.D.: standard deviation.

Table 3:

Validated reclassification scores of *Anopheles* species according to landmark-based and outline-based approaches, using *Cx. quinquefasciatus* as a putative outgroup.

Species	Percentage of reclassification scores (assigned/observed)	
	Landmark-based method	Outline-based method
<i>An. maculatus</i>	77% (147/191)	93% (186/200)
<i>An. sawadwongporni</i>	76% (32/42)	95% (41/43)
<i>An. pseudowillmori</i>	94% (34/36)	100% (36/36)
<i>Cx. quinquefasciatus</i>	100% (30/30)	100% (30/30)
Total	81% (243/299)	95% (293/309)