Comparison of the Efficiency of Landmark-Based and Semilandmark-Based Geometric Morphometrics to Diagnose Three *Anopheles* Species

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Abstract: In this study, our objective was comparison of the efficiency of Geometric Morphometrics (GM) to diagnose three *Anopheles* species including males and females of *An. barbirostris* s.l. van der Wulp *An. epiroticus* Linton and Harbach and *An. subpictus* s.l. Grassi in Thailand by using the Land Mark-based (LM), semil and mark-based (LMSL) methods of GM. The results of the size and shape analysis revealed clear similarities of both the LM and LMSL methods. The analysis of wing size based on centroid size in both the LM and LMSL methods indicated that the wing size patterns were different in all species except between *An. epiroticus* and *An. subpictus* s.l. females. All pairwise comparisons of wing shape based on Mahalanobis distance scores presented significant differences (permutation test: 1,000 cycles, p<0.05). Cross-validated reclassification scores based on assessing the accuracy of cluster recognition showed that the LMSL method was slightly more efficient than the LM method (96 vs. 91%). This study revealed that using the LMSL method can better separate the females and males of *An. barbirostris* s.l., *An. subpictus* s.l. and *An. epiroticus* than LM which can be useful in guiding the identification of correct vectors for malaria control.

Key words: Geometric morphometric, landmark and semi-landmark-based approach, *Anopheles barbirostris* s.l. van der Wulp, *Anopheles epiroticus* Linton and Harbach, *Anopheles subpictus* s.l. Grassi, malaria

INTRODUCTION

Malaria is a life-threatening disease that can present worldwide and which is caused by *Plasmodium* parasites transmitted by female *Anopheles* mosquitoes (Service, 2008). WHO. (2017) reported that there were 19 million cases and 435,000 deaths worldwide as a result of malaria. Generally, this disease occurs in the tropical and subtropical zones that are associated with *Anopheles* vectors (WHO., 2013). *Anopheles* mosquitoes belong to the subfamily Anophelinae which includes 488 formally recognized species (Tainchum *et al.*, 2015). Importantly, not all species of *Anopheles* mosquito are able to transmit malaria. *Anopheles* vectors and non-vectors are related in terms of vectorial capacity and the ability to transmit *Plasmodium* pathogens to humans (Brady *et al.*, 2016). The correct species identification of mosquito vectors is important for the selection of appropriate control methods. However, species identification based on morphological characteristics is quite difficult in some species with very similar morphology (WHO., 2007) or in the case of samples that

have been damaged in the field (Chaiphongpachara *et al.*, 2018). Molecular techniques are one of the methods available to help solve this problem but they are expensive and require sophisticated equipment to perform.

Geometric Morphometrics (GM) is a popular alternative approach used in entomological studies, especially in the species identification of mosquito vectors (Wilke *et al.*, 2016). Recently, GM has been used to identify three member species of *Anopheles* in the Maculatus group in Thailand including *An. maculatus An. sawadwongporni* and *An. pseudowillmori* (Chaiphongpachara *et al.*, 2019). In addition, GM has been applied to *An. barbirostris* s.l. and *An. subpictus* s.l. in the Ratchaburi province of Thailand Chaiphongpachara *et al.*, 2018). In both cases, GM was highly effective in identifying *Anopheles* mosquitoes. The Land Mark-based method (LM) is one of GM technique and is widely used, offering the advantages of being easy to use, speedy analysis and cheap cost (Dujardin, 2008). The coordinates of anatomical landmarks are typically used in LM analysis which often defines these landmarks

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spread across the wings of mosquitoes. Type 3 landmarks or semi landmarks (also called sliding semi landmarks) is a type of landmark that has been developed for the description of curved lines (Bookstein, 1997). Previous research has reported that landmarks combined with semilandmarks (also called Land Mark-and Semi-Landmark based method, LMSL) is more effective in identifying many species of tsetse flies than LM (Kaba *et al.*, 2017).

In this study, our objective was comparison of the efficiency of both the LM and LMSL GM methods to diagnose three *Anopheles* species including males and females of *An. barbirostris* s.l. van der Wulp, *An. epiroticus* Linton and Harbach and *An. subpictus* s.l. Grassi in Thailand. These three species of *Anopheles* mosquitoes are important vectors in malaria transmission in Southeast Asia. *An. barbirostris* s.l. is recognized as a significant malaria vector and has been found to be associated with infection with the protozoan parasite *Plasmodium* in many areas of Thailand (Rattanarithikul *et al.*, 1996). *An. epiroticus* is a secondary vector of malaria in Thailand found near coastal zones and associated with brackish water habitats (Sumruayphol *et al.*, 2010; Chaiphongpachara and Sumruayphol, 2017). To our knowledge, there is no report showing the relevance between *An. subpictus* s.l. and malaria in Thailand but for other countries such as Indonesia, India and Sri Lanka it is a major problem.

MATERIALS AND METHODS

Anopheles mosquito collection: Female and male adult *Anopheles* mosquitoes were captured using a Mosquito Magnet Independence Trap (Woodstream Corporation, Lititz, PA, USA) in two provinces in Thailand during June to August 2015. An. *barbirostris* s.l., *An. epiroticus* and *An. subpictus* s.l. were used in this study. *An. barbirostris* s.l. and *An. subpictus* s.l. were collected at border areas in Ratchaburi province (13°22'36.0"N, 99°16'34.9"E) while, *An. epiroticus* was collected at coastal areas in Samut Songkhram Province (13°24'32.52"N, 100°0'41.40"E). All mosquito collections were conducted from 18:00 until 06:00 and were performed once a week. *Anopheles* mosquitoes were sent to the laboratory of the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Center to identify species the following morning using the morphological keys (Rattanarithikul *et al.*, 2010).

Slide preparation: For GM analysis, the number of samples should be twice the number of landmarks. Therefore, 30 mosquitoes per species and sex were randomized and used for LM and LMSL analysis.
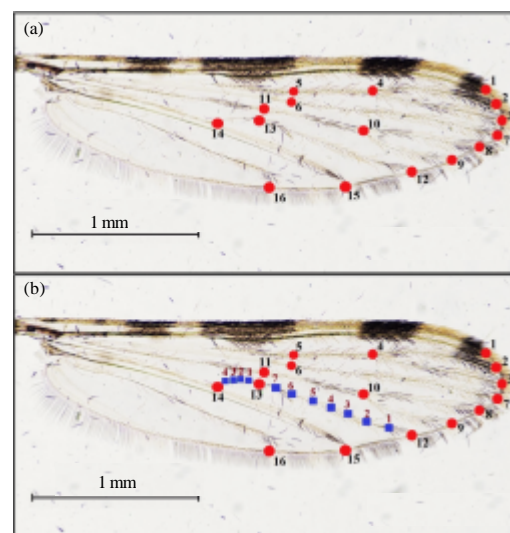

Fig. 1: Coordinates of landmarks and semilandmarks on mosquito wings for GM analysis: a) LM analysis and b) LMSL analysis

Complete left wings were removed from the collected mosquito bodies and mounted on microscope slides under a coverslip using Hoyer's solution as a permanent mounting medium. A Nikon DS-Ri1 SIGHT digital camera coupled to a Nikon SMZ745T stereomicroscope (Nikon, Tokyo, Japan) with 40x magnification was used to photograph wing images.

Both the LM and LMSL methods used the same analysis process but differ in their manners of determining the points of coordinates shown in Fig. 1a, b. Coordinates of 16 landmarks were selected and digitized for the LM method (Fig. 1a). In the LMSL methodcoordinates of 16 landmarks (same Landmarks in LM) combined with 11 semil and marks which were divided into two curved lines of the wing vein including seven semilandmarks between landmarks 12 and 13 and four semilandmarks between landmarks 13 and 14 were digitized (Fig. 1b).

Geometric morphometric analysis
Repeatability test: A repeatability test involved the repeated evaluation of the accuracy or measurement error of landmarks. The measurement error in this study was examined using the "Repeatability (R)" index (Arnqvist and Martensson, 1998). Landmark repeatability was tested in 10 wings per species in each sex selected randomly which were digitized twice by the same person.

Size analysis: The Centroid Size (CS) isometric estimator derived from coordinates was used to analyze the overall wing size among female and male *Anopheles* mosquitoes. CS is defined as "the square root of the summation of the

squared distances between the center of the configuration and each landmark individual" (Bookstein, 1991). Differences in wing CS in each *Anopheles* species were compared by a nonparametric method (1,000 cycles) with bonferroni correction at a risk level of 5%.

Shape analysis: Wing shape variables were computed using the generalized Procrustes analysis superimposition algorithm and the subsequent projection of the Procrustes residuals into an Euclidean space (Rohlf, 1999) with the resulting variables being partial warps. Discriminant analysis as a statistical method was performed to explore shape dissimilarity between species. Results from this were presented as a factor map by Canonical Variate Analysis (CVA) and were used to calculate the Mahalanobis distance scores. Wing shape differences in each species based on Mahalanobis distances were computed using a nonparametric method (1,000 cycles) with Bonferroni correction at a risk level of 5%.

Classification tests and morphological trees: A cross-validated reclassification test was used to assess the accuracy of cluster recognition based on Mahalanobis distance scores. For assessment, each mosquito wing was reclassified according to its wing shape similarity within each group. Morphological trees of both the LM and LMSL methods were established using the neighbor-joining method to investigate the nearness of wing shape in each species. Thirty male and female *Aedes aegypti* were used as an out-group in the morphological trees.

Software: The CLIC package as a GM software was used for LM and LMSL analysis in this study and is freely available at http://xyom-clic.eu/. The neighbor-joining trees were developed using the R Software (R Foundation, Vienna, Austria) which is freely available at https://cran.r-project.org/.

RESULTS AND DISCUSSION

Repeatability: The comparison of two repeated sets of measurements for the same wing images showed good repeatability scores for wing size based on CS (R = 0.98 for males and 0.97 for females) and wing shape based on relative warps (R = 0.95 for males and 0.94 for females).

Wing size: The morphometric analysis of wing CS for both the LM and LMSL methods yielded the same results. The mosquito with the largest wings was the *An. barbirostris* s.l. female and that with the smallest wings was the *An. subpictus* s.l. male. Wing CS variations between species and sexes of each *Anopheles* species in both the LM and LMSL methods are shown in Fig. 2a, b. Sexual size dimorphism was different among all species with the females generally being larger than the males. The mean wing CS values of female and male *Anopheles* mosquitoes were significantly different in all pairwise comparisons (p<0.05, Table 1).

Wing shape: The factor maps of the discriminant analysis generated from CVA (Fig. 3) for exploring shape dissimilarities between species and sexes revealed that almost all of the specimens could be separated into distinct groups, especially, when considering the factor map of LMSL. All pairwise comparisons of wing shape based on Mahalanobis distance scores presented significant differences (permutation test: 1,000 cycles, p<0.05, Table 2).

Accuracy of cluster recognition and neighbor-joining trees: The percentages of correctly classified samples obtained from the cross-validation reclassification test revealed high scores for both methods, specifically, 91% for the LM method and 96% for the LMSL method (Table 3). The investigations of nearness of wing shape in each species and sex were visualized by the neighbor
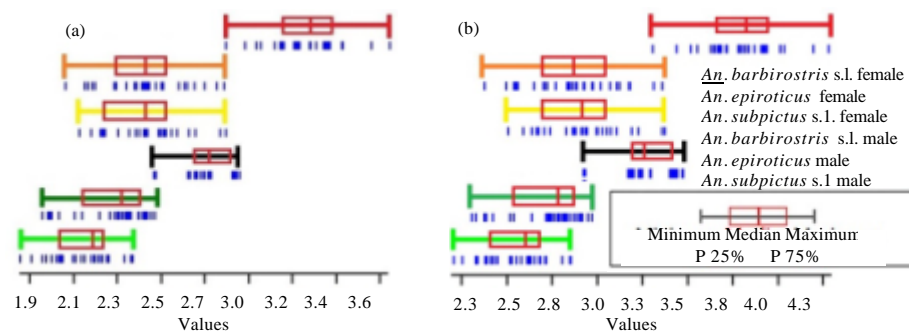

Fig. 2: Wing CS variation between species and sexes in both: a) LM and LMS b) methods (units are pixels converted to millimeters (mm), shown as quantile boxes. Each box shows the group median (25th and 75th percentiles) and the vertical bars under the boxes represent the wings
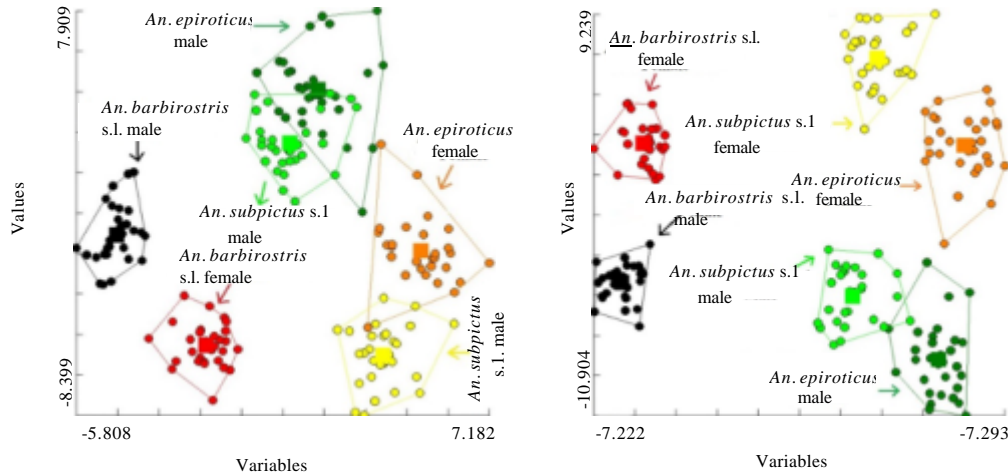
Fig. 3: Discriminant analysis of both: a) LM and b) LMSL methods. The horizontal axis is the first discriminant factor and the vertical axis is the second discriminant factor

Table 1: Wing CS comparisons between species and sexes of *Anopheles* mosquitoes

	Mean±SD (Min-Max)	
Species	LM	LMSL
An. barbirostris s.l. female	3.22±0.03 (2.84-3.61)[a]	3.84±0.04(3.36-4.30)[a]
An. epiroticus female	2.44±0.04 (2.07-2.82)[b]	2.94±0.06(2.46-3.42)[b]
An. subpictus s.l. female	2.45±0.03 (2.14-2.83)[b]	2.95±0.04(2.56-3.39)[b]
An. barbirostris s.l. male	2.78±0.01 (2.52-2.92)[c]	3.32±0.02(2.99-3.50)[c]
An. epiroticus male	2.29±0.03 (1.97-2.50)[d]	2.76±0.04(2.36-3.01)[d]
An. subpictus s.l. male	2.17±0.02 (1.88-2.40)[e]	2.61±0.03(2.26-2.89)[e]

LM = Landmark-based Method, LMSL = Semilandmark-based Method, SD = Standard Deviation, Min = Minimum, Max = Maximum. Different superscript letters in each row indicate statistical differences at p<0.05

Table 2: Comparisons of Mahalanobis distance scores between species and sexes of *Anopheles* mosquitoes

Methods/species	*An. barbirostris* male	*An. epiroticus* female	*An. subpictus* female	*An. barbirostris* male	*An.epiroticus* male	*An subpictus* male
LM						
An. barbirostris s.l. female	-					
An. epiroticus	9.36*	-				
An. subpictus s.l. female	7.73*	5.05*	-			
An. barbirostris s.l. male	5.70*	12.33*	11.52*	-		
An. epiroticus	9.55*	7.13*	9.32*	9.61*	-	
An. subpictus s.l. male	7.91*	6.92*	8.05*	7.77*	3.85*	-
LMSL						
An. barbirostris s.l. female	-					
An. epiroticus	16.73*	-				
An. subpictus s.l. female	13.29*	7.27*	-			
An. barbirostris s.l. male	6.53*	18.94*	16.39*	-		
An. epiroticus	17.33*	9.49*	12.63*	17.17*	-	
An. subpictus s.l. male	12.80*	9.51*	9.77*	12.59*	7.61*	-

LM = Landmark-based Method, LMSL = Semilandmark-based Method.*indicates statistical significance at p<0.05

Table 3: Percentages of cross-validated reclassification scores based on wing shape similarities

	Accuracy of classification (assigned/observed)	
Species	LM(%)	LMSL(%)
An. barbirostris s.l. female	100 (30/30)	100 (30/30)
An. epiroticus female	86 (26/30)	86 (26/30)
An. subpictus s.l. female	90 (27/30)	96 (29/30)
An. barbirostris s.l. male	100 (30/30)	100 (30/30)
An. epiroticus male	80 (24/30)	90 (27/30)
An. subpictus s.l. male	86 (26/30)	100 (30/30)
Total	91(163/180)	96(172/180)

% = Percentage, LM = Landmark-based Method, LMSL = Semilandmark-based Method

joining trees based on the Procrustes distances. Both the LM and LMSL methods led to the same morphological tree pattern which located *An. barbirostris* s.l. females, *An. epiroticus* females, *An. subpictus* s.l. females, *An. epiroticus* males and *An. subpictus* s.l. males into the same cluster but separated out *An. barbirostris* s.l. males (Fig. 4). Female and male *Aedes aegypti* were additionally clearly separated from all other *Anopheles* mosquitoes.
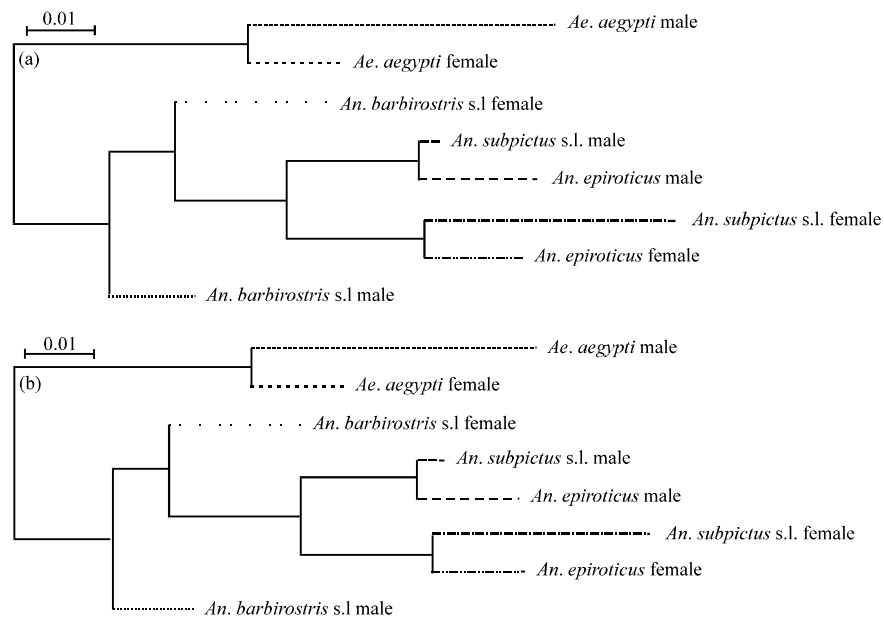
Fig. 4: Neighbor-joining trees of both: a) LM and b) LMSL GM method

The present research evaluated and compared the efficiency of GM between the LM and LMSL GM methods to diagnose three female and male *Anopheles* species in Thailand including *An. barbirostris* s.l., *An. epiroticus* and *An. subpictus* s.l. mosquitoes. The results of the size and shape analysis reveal clear similarities of both the LM and LMSL methods.

The analysis of wing size based on CS using both the LM and LMSL methods indicated wing size patterns were almost all different in all species except in the pairing of *An. epiroticus* and *An. subpictus* s.l. females. However, the consideration of wing size is not a good primary way for diagnosing species because it is not stable and is highly sensitive to the environment. The influence of the surrounding environment during the larval stage such as the quantity or quality of food and temperature affects adult mosquitoes directly (Jirakanjanakit *et al.*, 2017). Most wing size analysis data are often used to study morphological variations in different environments, rather than being used for mosquito species identification (Lorenz *et al.*, 2017). Previous research has been conducted on the influence of environmental heterogeneity on *An. darlingi* in Northwest Colombia and found that wing size differences may reflect plastic responses rather than genetic divergence due to low genetic differentiation and high gene flow in mosquito populations (Altamiranda-Saavedra *et al.*, 2017).

The wing shape of mosquitoes has been recognized as an important evidence of microevolution and identity of each species separate from genetic expression

(Suesdek, 2018). Thus, wing shape is often used to diagnose species of mosquito vectors using by GM. The results of the wing shape evaluation of both GM methods in the present investigation indicated significant differences exist in wing shape in all species and sexes. These results were consistent with those of other recent research in Thailand that found that GM has the potential to separate *An. barbirostris* s.l. and *An. subpictus* s.l. from one another (Chaiphongpachara, 2018). Cross-validated reclassification scores based on assessing the accuracy of cluster recognition have shown that the LMSL method is slightly more efficient than the LM method (96 vs. 91%). These analysis findings suggest that the use of landmarks in combination with the analysis of the important curved lines of the wing vein using the LMSL method can increase the efficiency of stratifying *An. barbirostris* s.l. *An. subpictus* s.l. and *An. epiroticus* females and males. This heightened efficiency for diagnosing species may be due to the increasing coordinates for analysis and the fact that the positioning of these curve lines constitutes a unique characteristic that helps to separate species from each other. This is consistent with previous research that found that LMSL has more efficiency on species identification than LM in many species of tsetse flies (Kaba *et al.*, 2017).

CONCLUSION

The GM technique is a tool to help diagnose certain species of mosquitoes that are difficult to separate from

one another using morphological characteristics alone. LM is one method of GM that is commonly used to identify mosquito vectors because of its ease and quickness of use as well as cheapness. This study revealed that using the LMSL can better separate *An. barbirostris* s.l. *An. subpictus* s.l. and *An. epiroticus* females and males than LM. This information will useful for guiding the identification of correct vectors for malaria control.

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