JOURNAL OF ADVANCED VETERINARY AND ANIMAL RESEARCH
ISSN 2311-7710 (Electronic)
http://doi.org/10.5455/javar.2019.f345
A periodical of the Network for the Veterinarians of Bangladesh (BDvetNET)

September 2019
VOL 6, NO. 3, PAGES 278–283

ORIGINAL ARTICLE

Effectiveness of landmark- and semi-landmark-based geometric morphometric to identify four species of *Culex* mosquitoes in Thailand

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ABSTRACT

Objective: This research aims to study the effectiveness of landmark- and semi-landmark-based geometric morphometric (LMSL) in the identification of four *Culex* vectors, namely, *C. quinquefasciatus, C. visnui, C. sitiens,* and *C. whitmorei* in Thailand and also compared the potential between LMSL and the landmark-based geometric morphometric method (LM).
Materials and Methods: The overall size of the wing sample was estimated by the centroid size. Shape variables were computed as principal components of the "partial warp" calculated after generalized procrustes analysis of raw coordinates. Discriminant analysis of the canonical variables performed to explore the shape dissimilarity between *Culex* species has been shown as a factor map and to calculate the Mahalanobis distance. Size and shape differences based on pairwise Mahalanobis distances were tested using non-parametric methods (1,000 cycles) with Bonferroni correction at a *p*-value of <0.05.
Results: A total of 120 individuals were used that were divided into 30 individuals per *Culex* species. The mean CS of *C. sitiens* had the largest wings followed by *C. visnui*, *C. quinquefasciatus*, and *C. whitmorei* in LM and LMSM. The patterns of statistical difference in CS of both methods were similar and wing shapes among *Culex* species were different based on a comparison of pairwise Mahalanobis distances ($p < 0.05$) in both methods. For the cross-validated reclassification test, LM provided *Culex* species separation ranging from 54% to 84% and 51% to 93% for LMSM.
Conclusion: Thus, LMSM is another option to use for the identification in mosquito vectors that have a curved line on the wing specific to the species.

ARTICLE HISTORY
Received January 11, 2019
Revised January 30, 2019
Accepted February16, 2019
Published June 17, 2019

KEYWORDS
Geometric morphometrics, *Culex quinquefasciatus*, *Culex visnui*, *Culex sitiens*, *Culex whitmorei*

Introduction

Mosquitoes are small insects belonging to the order Diptera, which are capable of transmitting pathogens to humans, such as Zika virus, chikungunya virus, yellow fever, dengue fever, Japanese encephalitis virus, and filarial nematode [1]. *Culex* mosquitoes are one of the medically important genera, which have members most widespread throughout the world [2]. Japanese encephalitis and lymphatic filariasis are important tropical diseases that often have *Culex* species as vectors [3]. Twenty-four countries in the South-East Asia and Western Pacific regions are endemic areas for Japanese encephalitis, and more than 3 billion people are at risk of infection, while for lymphatic filariasis, 856 million people in 52 countries worldwide remain at risk of infection [4].

Thailand is one of the countries facing the problem of *Culex*-borne diseases, including Japanese encephalitis and lymphatic filariasis. One effective way to control mosquito-borne diseases is to reduce the mosquito population in the area [5]. There are many ways for mosquito control, which must be suitably selected with the target species of vectors. It is often difficult to identify mosquito species as they have similar morphological characteristics in some species.

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How to cite: Chaiphongpachara T, Laojun S. Effectiveness of landmark- and semi-landmark-based geometric morphometric to identify four species of *Culex* mosquitoes in Thailand. J Adv Vet Anim Res 2019; 6(3):278–283.

Geometric morphometrics (GM) is a powerful tool to use for species identification, especially arthropods [6,7]. Previous research has applied GM for identification between species to eggs of Triatominae [8], trombiculid mites [9], tsetse flies [10], mosquitoes [11,12]. Currently, mosquito wing is considered to help identification by GM particularly a landmark-based method because their wing is the perfect organ for GM analysis [6,7]. Although the landmark-based GM method (LM) is an effective technique of identifying some mosquito species, it is not perfect because it is difficult to analyze the curve, which may be the specific location of the target species. The landmark- and semi-landmark-based GM method (LMSL) is one GM technique that was developed to improve the weaknesses of LM in curve position analysis. LMSL is a combination of landmark and semi-landmark for analysis [10]. However, the researches that have applied LMSL are relatively few, which may affect the actual use in other areas.

Thus, this research aims to study the effectiveness of LMSL in the identification of four *Culex* vectors in Thailand, namely, *C. quinquefasciatus*, *C. visnui, C. sitiens*, and *C. whitmorei*. *Culex quinquefasciatus* has been considered as the Japanese encephalitis virus and filarial nematode vector, *C. visnui* and *C. sitiens* are Japanese encephalitis virus vectors, while *C. whitmorei* is a filarial nematode vector. It also compared the potential between LMSL and LM as a guideline for further application.

Materials and Methods

Sample collection

In this study, four species of female *Culex* mosquitoes, as important vectors in Thailand, were used, namely, *C. quinquefasciatus*, *C. visnui*, *C. sitiens*, and *C. whitmorei*. We collected three species, including *C. quinquefasciatus*, *C. visnui*, and *C. whitmorei* from Nam Nak village (13°22′36.0′′N, 99°16′34.9′′E) in Ratchaburi Province, Thailand and *Cx. sitiens* from the coastal community area of Samut Songkhram Province, Thailand (13°24'34.3"N, 100°00'52.9"E) during August 2015 once a week. Independent mosquito traps (Woodstream Corporation, USA) were used for adult female collection at night (6.00 PM–6.00 AM). Every morning (6.00 AM), mosquitoes were taken from traps and sent to the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Center for species identification by stereomicroscopic observation based on morphologic characters using the illustrated keys to the mosquitoes in Thailand [13].

Wing preparation

Thirty-three mosquitoes were selected randomly per *Culex* species for analyses (a total of 120 individuals, including 30 individuals of *C. quinquefasciatus*, 30 individuals of *C. visnui*, 30 individuals of *C. sitiens*, and 30 individuals of *C. whitmorei*). The right wing of each adult *Culex* mosquito was removed from the thorax and mounted on a microscope slide under a 0.08–0.12 mm coverslip using Hoyer's medium. A digital camera connected to a Nikon SMZ745T stereomicroscope (Nikon Corp., Tokyo, Japan) under 40× magnification was used to photograph wing images.

Landmark-based method (LM) and Landmark- and semi-landmark-based method (LMSL)

In this study, we used two different technical GM methods, namely, LM and LMSL, to compare the effectiveness of both methods. Both methods are similar for analysis and calculation but different at anatomical points. For LM, 11 landmarks in Fig. 1A were digitized in each wing image. For LMSL, 11 landmarks (same positions landmarks in LM) combined with 12 semi-landmarks at the curved line of the wing vein between landmarks 9 and 10 (Fig. 1B) were digitized. After that, the repeatability index was calculated for evaluating the accuracy of landmarks and semi-landmarks of both methods by comparison of repeat plots [14].

The overall size of the wing sample was estimated by the CS, which is the square root of the sum of the squared distances from the centroid to each landmark [15], and CS variations of *Culex* species were shown by quantile boxes. Differences of mean CS among species of both methods were compared using non-parametric methods (1,000

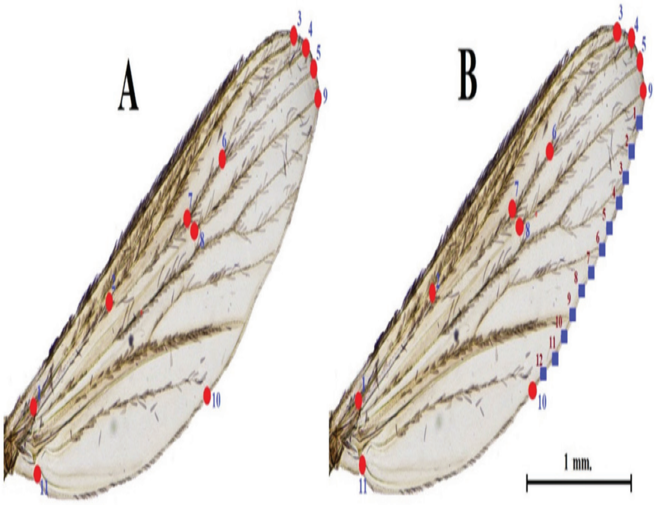

Figure 1. Positions of landmarks and semi-landmarks. (A) 11 landmarks for LM analysis and (B) 11 landmarks combined with 12 semi-landmarks for LMSL analysis..

cycles) and relevant Bonferroni correction. The significance level was set at $p < 0.05$.

Shape variables were computed as principal components of the "partial warp" calculated after generalized procrustes analysis of raw coordinates. Discriminant analysis of the canonical variables performed to explore the shape dissimilarity between *Culex* species has been shown as a factor map and to calculate the Mahalanobis distance. Shape differences based on pairwise Mahalanobis distances were tested using non-parametric methods (1,000 cycles) with Bonferroni correction at a p-value of <0.05.

Each wing individual was then reclassified to test the accuracy of *Culex* species classification obtained using the cross-validated reclassification test based on Mahalanobis distance scores. Phenetic trees of both LM and LMSM were performed using the Neighbor-Joining method for assessing the closeness of wing shapes in each species. Thirty individuals of female *Aedes aegypti* were used as an out-group.

Software

The software used to analyze LM and LMSL in this study was the CLIC package, which is freely available at http://xyom-clic.eu/. Phenetic trees of both GM methods in this study were performed using R software freely available at https://cran r-project.org/.

Results and Discussion

A total of 120 individuals were used that divided into 30 individuals per *Culex* species, including *C. quinquefasciatus*, *C. visnui*, *C. sitiens*, and *C. whitmorei.* The repeatability test of both showed good repeatability for CS (0.96 for LM and 0.94 for LMSM) and the relative warps (0.93 for LM and 0.91 for LMSM).

Wing size variation among Culex species

CS variations among *Culex* species of both methods are detailed in Fig. 2. The mean CS of *C. sitiens* had the largest wings followed by *C. visnui*, *C. quinquefasciatus*, and *C. whitmorei* in LM and LMSM. The patterns of statistical difference in CS of both methods were similar as shown in Table 1.

Wing shape variation among Culex species

Shape variations among *Culex* species were shown in Fig.3A for LM and Fig. 3B for LMSL. LM and LMSM discriminant analysis based on wing shape showed that individuals clustered into distinct groups in factor maps according to each *Culex* species (Fig. 4). All pairwise wing shapes were different among *Culex* species in both methods based

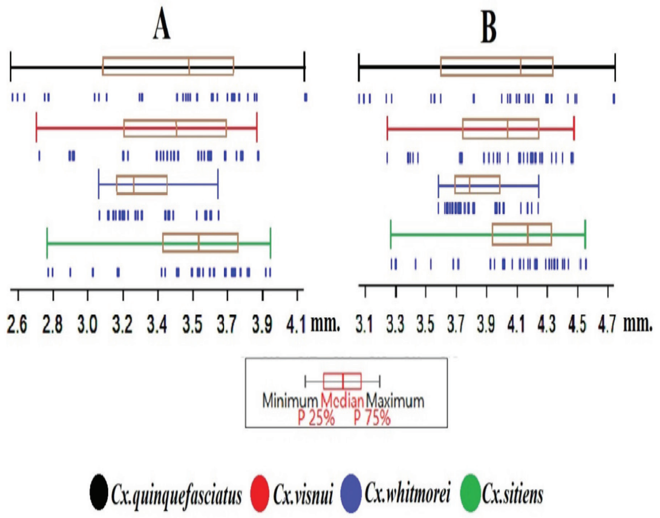

Figure 2. Wing CS variation between *Culex* species of (A) LM and (B) LMSL. Each box shows the median as a line in the middle and the quartiles (25th and 75th percentiles)

Table 1. Wing CS comparisons between Culex species.

***Culex* species**	**Mean ± Standard deviation (mm.)**	
	LM	**LMSL**
Cx. quinquefasciatus	$3.42 \pm 0.17^{a,b}$	$3.95 \pm 0.22^{a,b}$
Cx. visnui	3.43 ± 0.08^{a}	3.96 ± 0.10^{a}
Cx. whitmorei	3.29 ± 0.03^{b}	3.82 ± 0.03^{b}
Cx. sitiens	3.51 ± 0.07^{a}	4.03 ± 0.10^{a}

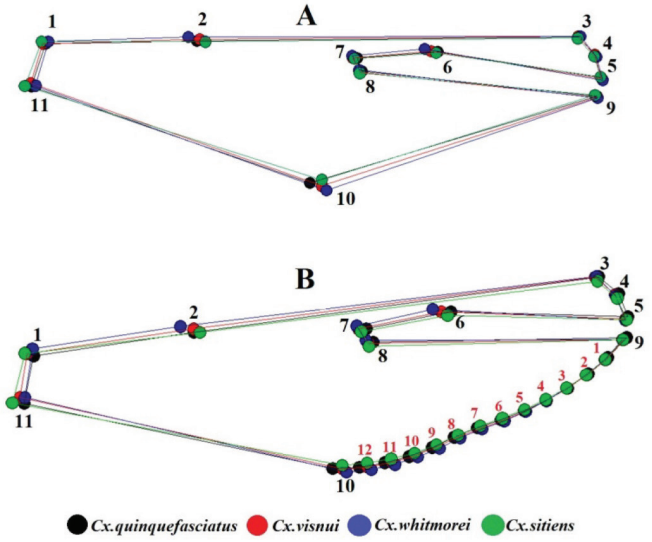

Figure 3. Superimposition of mean landmark configurations between *Culex* species in (A) LM and (B) LMSM.

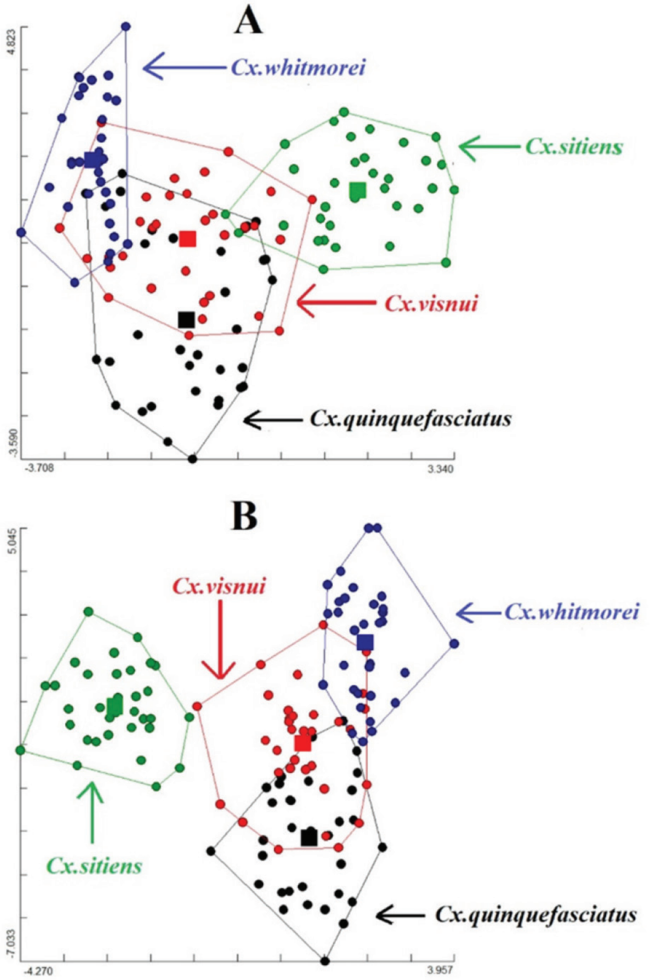

Figure 4. (A) LM discriminant analysis and (B) LMSM discriminant analysis. Factor map of the two discriminant factors (DFs) derived from final shape variables. Each point represents a Culex individual. The horizontal axis is the first DF; the vertical axis is the second DF.

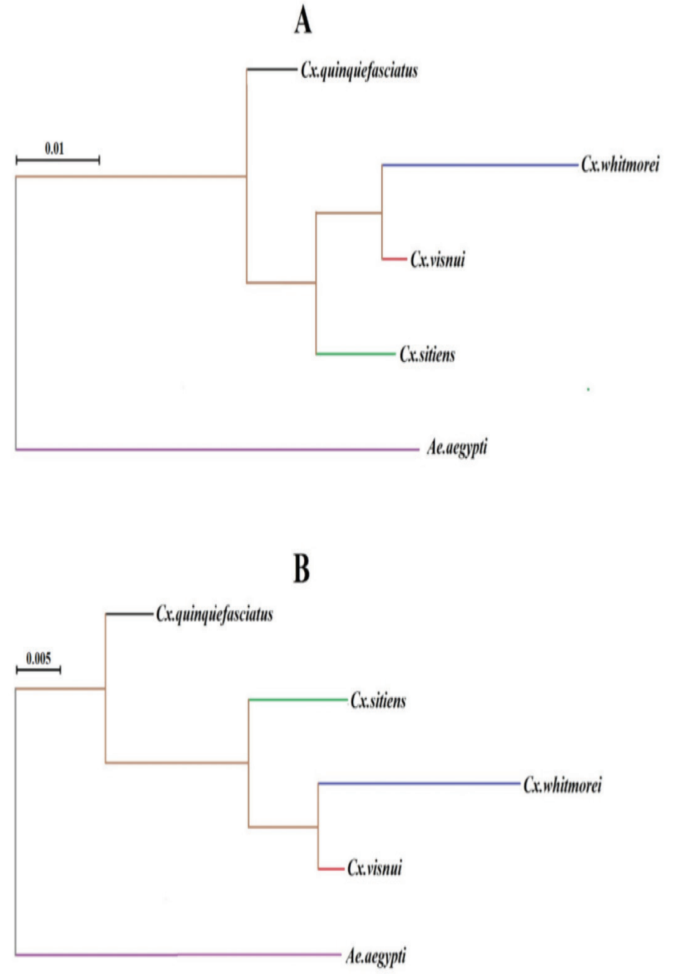

Figure 5. Phenetic trees for (A) LM and (B) LMSM analysis of *Cx. quinquefasciatus, Cx. visnui, Cx. sitiens and Cx. whitmorei.*

Table 2. Statistical analyses of pairwise Mahalanobis distances between Culex species.

Methods	*Culex* species	*Cx. quinquefasciatus*	*Cx. visnui*	*Cx. whitmorei*	*Cx. sitiens*
LM	*Cx. quinquefasciatus*	-			
	Cx. visnui	2.44*	-		
	Cx. whitmorei	3.18*	2.95*	-	
	Cx. sitiens	3.96*	3.80*	5.18*	-
LMSL	*Cx. quinquefasciatus*	-			
	Cx. visnui	3.09*	-		
	Cx. whitmorei	4.03*	3.50*	-	
	Cx. sitiens	5.99*	5.63*	7.08*	-

*Statistical differences at $p < 0.05$.

on a comparison of pairwise Mahalanobis distances ($p < 0.05$, Table 2). For the cross-validated reclassification test, LM provided *Culex* species separations ranging from 54% to 84% and 51% to 93% for LMSM. Patterns of the phenetic trees of both methods were shown in Fig. 5, which located *C. visnui*, *C. sitiens*, and *C. whitmorei* into the same cluster and separated *C. quinquefasciatus*. *A. aegypti* as an out-group was clearly separated from *Culex* mosquitoes.

Although characters used in the morphological analysis are the gold standard method for mosquito species identification, *Culex* species are difficult to distinguish in the field due to target organ damage by mosquito traps such as CDC light trap [16] and black light (UV) trap [17]. Geometric morphometrics are new techniques that have been used as an alternative for identification [18,19]. In previous research, we have used landmark- and outline-based GM to distinguish seven species of mosquito vectors, namely, *Anopheles barbirostris*, *A. subpictus*, *C. quinquefasciatus*, *C. vishnui*, *C. whitmorei, A. aegypti*, and *A. albopictus* in Ratchaburi Province, Thailand and found that the identification of *Culex* mosquitoes was poor in *C. quinquefasciatus* and *C. vishnui* [11]. In this study, LMSM was applied to determine the efficacy of the discriminant in the four species of *Culex* mosquitoes, which was found to be effective for identification.

By comparison of the wing size based on CS values of the two GM methods, we found that they have the same appearance patterns. Corresponding to the previous research, outline-based GM analysis (digitized contour of the lower section of wing for analysis) of mosquito vectors in Ratchaburi Province, Thailand reported that *C. visnui* was largest, followed by *C. quinquefasciatus* and *C. whitmorei*. *Culex sitiens* had the largest size among *Culex* species in this study but this result cannot be linked to this mosquito species in other areas because size is commonly subject to plasticity and ability to adapt to different environmental conditions [15,20]. Although the analysis of size has shown statistical differences in some species in LM and LMSM, the shape is considered more suitable for species discrimination than the size [21,12].

Shape has been accepted as having less environmental variance than the size [6,22]. In addition, the wing shape of a mosquito was sensitive to detect microevolution from the genetic back ground and geographical variation [23–26]. The results of the shape analysis had shown the statistical difference in all the species of LM and LMSM, in which both methods have the potential to distinguish *Culex* mosquitoes. Likewise, the patterns of the phenetic trees were expressed similarly between LM and LMSM, which shows similar shape-estimation results of both GM methods. A comparison of the identification efficiency of both methods based on cross-validated reclassification scores revealed that the potential for identification depends on the species of mosquitoes. This study has indicated that LMSM can identify *C. quinquefasciatus* and *C. sitiens* better than LM, which showed that the curves used in this study were identifiable characteristics of both species.

Conclusion

LMSM is one GM that can help to identify species of mosquitoes. This research has revealed its effectiveness in identifying four *Culex* mosquito vectors in Thailand. It has a higher potential for some *Culex* species than LM, which is a popular method. Thus, this method is another option to use for identification in mosquito vectors that have a curved line on the wing specific to the species.

Acknowledgments

This study was supported by the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Center.

Conflict of Interest

The authors declared that they have no conflict of interests.

Authors' contribution

Sedthapong Laojun analyzed the data and Tanawat Chaiphongpachara wrote the manuscript.

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