Geometric morphometric analysis of interspecific size and shape variation in *Culex whitmorei* and *Cx. gelidus* (Diptera: Culicidae) in Thailand

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Culex whitmorei Giles and *Cx. gelidus* Theobald (Diptera: Culicidae) are important vectors of Japanese encephalitis virus in Southeast Asian countries, including Thailand. In some parts of Thailand, *Cx. whitmorei* and *Cx. gelidus* can be found in the same area due to their similar ground water habitats. In addition, both mosquito species have similar morphological characteristics, an issue which can cause confusion in identification and lead to errors in distinguishing both species. This research evaluated the effectiveness of geometric morphometric to separate female *Cx. whitmorei* and *Cx. gelidus* in Thailand. The results of overall wing size analysis based on centroid size demonstrated that the wings of *Cx. whitmorei* were larger than *Cx. gelidus* and found statistically significant differences between species ($p < 0.05$), while wing shape of both species was different based on pairwise Mahalanobis distances ($p < 0.05$). *Culex* species-identification reliability after the cross-validated classification test was 100% for *Cx. whitmorei* and 96% for *Cx. gelidus*. Analyses in this study illustrate the significant potential of using the geometry of morphometric factors for separating both *Cx. whitmorei* and *Cx. gelidus*. This success provides important information with respect to separation of both mosquitoes in other areas. It also supports the effectiveness of using the geometric morphometric in the identification of mosquito vectors in the field.

**Keywords: Geometric morphometric, landmark-based method*, Culex whitmorei, Culex gelidus*

INTRODUCTION

Japanese encephalitis *(*JE*)* is an important public health problem in many Asian countries, with an estimated 68,000 cases per year caused by the Japanese encephalitis virus (JEV) (Misra and Kalita, 2010; World Health Organization, 2016). Most infections of this virus are mild or without apparent symptoms, but approximately < 1% of infections resulting in clinical illness, with the case fatality rate being as high as 30% among those with disease symptoms (Misra and Kalita, 2010; Arshad et al., 2013). JEV

is an arbovirus in the family Flaviviridae related to dengue, yellow fever, and West Nile viruses, and is transmitted to humans by mosquitoes, particularly the *Culex* genera (Killick-Kendrick, 1996). The transmission cycle of this virus has a relationship between the mosquito vectors and the amplifying vertebrate hosts, including wading birds of the family *Ardeidae* and swine (Nemeth et al., 2012). *Cx. tritaeniorhynchus* Giles is the primary vector of JEV and is distributed across South-East Asia and other tropical areas, also extending into the Middle East and Africa and has

recently been reported in Europe (Longbottom et al., 2017). However, other *Culex* mosquito species have been implicated as primary or secondary vectors of JEV, such as *Cx. whitmorei* Giles and *Cx. gelidus* Theobald (Rattanarithikul et al., 2005; Longbottom et al., 2017).

Cx. whitmorei and *Cx. gelidus* (Diptera: Culicidae) are important vectors of JEV in Southeast Asia, including Thailand, which is one of the countries where JE cases occur annually. In some areas in Thailand, *Cx. whitmorei* and *Cx. gelidus* can be found in the same area due to the presence of similar ground water habitats such as swamps, ditches, pits, stream margins and rice fields (Rattanarithikul et al., 2005). In addition, both mosquito species have similar morphological characteristics, which sometimes cause confusion in identification and lead to errors in distinguishing both species.

The identification of these two species is often based on morphological examination of the scutum and legs. The distinctive morphology of *Cx. whitmorei* comprises "a patch of white scales reaching the prescutellar area and anterior surfaces of the femora speckled with pale scales", while *Cx. gelidus* is distinguished by "its patch of white scales not reaching the prescutellar area, with anterior surfaces of the femora being entirely dark" (Rattanarithikul et al., 2005). Often, these important morphological characteristics of mosquito samples in the field are destroyed and damaged via collection from mosquito traps (Chaiphongpachara T, 2018; Chaiphongpachara et al., 2018). This problem requires other alternative methods to help support the prevention of morphological identification errors.

Currently, geometric morphometrics (GM) are one of the alternative methods that has been accepted for species identification support of mosquito vectors (Rohlf, 2002; Lorenz et al., 2017; Dujardin, 2008). Recently, landmark- and outline-based GM techniques have been used to separate seven species within three genera of mosquito vectors — *Anopheles barbirostris, An. subpictus, Cx. quinquefasciatus, Cx. vishnui, Cx. whitmorei, Aedes aegypti*, and *Ae. albopictus* — in Huay Nam Nak village, Ratchaburi Province, Thailand; these approaches have also been found to be particularly effective in species such as *Cx. whitmorei* (Chaiphongpachara, 2018). Therefore, this research evaluated GM's effectiveness for separating *Cx. whitmorei* and *Cx. gelidus* as vectors of JEV in Thailand. This work was intended to assist in solving the problem of classification errors between these two types of mosquitoes that may occur in the field.

MATERIALS AND METHODS

Mosquito collection and sample preparation

Cx. whitmorei in this study were collected from Huay Nam Nak village (13°22′36.0″N, 99°16′34.9″E) in Ratchaburi Province, Thailand, while *Cx. gelidus* were collected from Samut Songkhram Province (13°24'32.52"N 100° 0'41.40"E) using Mosquito Magnet Traps (Woodstream Corporation, USA) during June to August 2015. All mosquito samples in the field were sent to the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram provincial education center to carry out species identification. Female *Cx. whitmorei* and *Cx. gelidus* as vectors of JEV were identified under a Nikon AZ 100M stereomicroscope (Nikon Corp., Tokyo, Japan) based on diagnostic morphological characteristics using the illustrated keys for the mosquitoes of Thailand (Rattanarithikul et al., 2005;).

After mosquito identification, we selected the female *Cx. whitmorei* and *Cx. gelidus* with the right wing completely undamaged for GM analysis. The right wing of each *Culex* individual was dissected and mounted on a microscope slide and coverslip using Hoyer's solution. Mounted wing slides were photographed using a Nikon DS- Ri1 SIGHT digital camera connected to a Nikon AZ 100M stereomicroscope at 40× magnification with a 1-mm scale bar.

Wing geometric morphometric analysis

In this study, digitization of landmarks, shape and size analyses were performed using CLIC Software at https://xyom.io. Five modules of CLIC Software were used – COO, MOG, TET, VAR, and PAD. Data obtained from *Culex* wings were arranged and scaled into millimeters in the TET module prior to landmark collection. After that, 12 landmarks were digitized (Figure 1) to use for landmark-based GM analyses in the COO module.

Size analysis

The wing size of *Culex* mosquitoes was estimated by the centroid size (CS)(Bookstein, 1991), while CS variation of female *Cx. whitmorei* and *Cx. gelidus* was determined using quantile boxes in MOG.
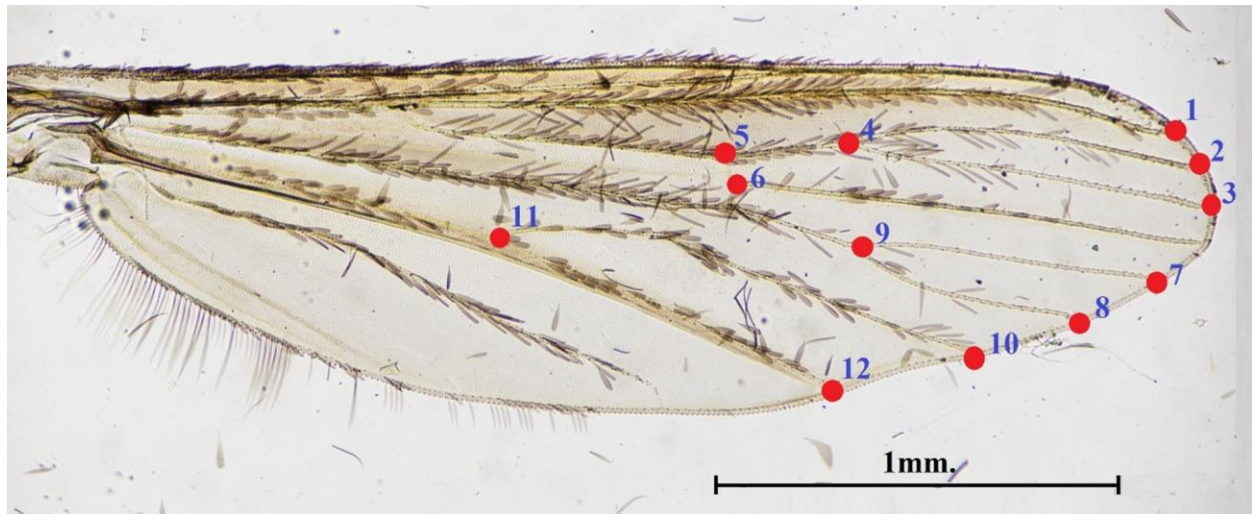
Figure 1. Twelve landmarks on the *Culex* wing for GM analysis.

Statistical significance of wing CS differences between species was tested in the VAR module using non-parametric method (1,000 permutations) with Bonferroni correction, in which if $p < 0.05$, the differences were considered to be significant.

Shape analysis

Procrustes superimposition is the initial procedure in analysis for wing shape calculations. Partial warp (PW) scores were used as shape variables, which were processed by standard multivariate analysis after Procrustes superimposition. The principal components of the PW (i.e., relative warps [RW]) were computed to visualize the morphospace for group segregation of *Cx. whitmorei* and *Cx. gelidus*. Discriminant analysis which used as input variables for the RW was performed to explore the level of different shapes between species and was illustrated by factor maps. These processes were performed in the MOG module. The difference of wing shape between *Cx. whitmorei* and *Cx. gelidus* based on Mahalanobis distances (generalized distance) was computed by non-parametric methods (1,000 permutations) with Bonferroni correction ($p < 0.05$). Finally, a cross-validated classification test was used for testing the accuracy of cluster recognition among species. Both statistical and cross-validated classification tests were performed in the POD module.

RESULTS

Wing size

Culex samples selected in the analysis comprised 50 individuals, divided into 25 individuals per species (25 individuals for both *Cx. whitmorei* and *Cx. gelidus*). The results of the wing size analysis based on CS, wing CS variation (Figure 2), and the mean values of both species of *Culex* mosquitoes demonstrated that *Cx. whitmorei* were larger than *Cx. gelidus* (1.97 mm VS. 1.82 mm in Table 1). In addition, both *Cx. whitmorei* and *Cx. gelidus* had statistically different wing CS at $p < 0.05$ (Table 1).

Wing shape

Superimposition of the mean landmark configurations between *Cx. whitmorei* and *Cx. gelidus* showed variation of both species for each landmark (Figure 3).

The morphospace calculated from principal components indicates the overlay between the groups (Figure 4A), while the discriminant analysis factor map clearly demonstrated segregation between *Cx. whitmorei* and *Cx. gelidus* (Figure 4B). Wing shapes of both species were different based on pairwise Mahalanobis distances ($p < 0.05$, Table 2). *Culex* species-identification reliability after the cross-validated classification test was 100% for *Cx. whitmorei* and 96% for *Cx. gelidus*.

Figure 2. Wing CS variation (in mm) between *Cx. whitmorei* and *Cx. gelidus*. The box denotes the median as a line across the middle and quartiles (25th and 75th percentiles).

Table 1. Statistical significance of mean wing CS differences between species.

Culex mosquito	Mean ± SD (mm)	Min-Max (mm)	*p*-value
Cx. whitmorei	1.97 ± 0.01	1.82–2.13	< 0.01
Cx. gelidus	1.82 ± 0.01	1.55–2.04	

Table 2. Statistical analyses of Mahalanobis distances between *Cx. whitmorei* and *Cx. gelidus*.

Species	Mahalanobis distances scores	p-value
Cx. whitmorei	6.27	< 0.01
Cx. gelidus		

Table 3. Results of cross-validated classification in *Cx. whitmorei* and *Cx. gelidus*.

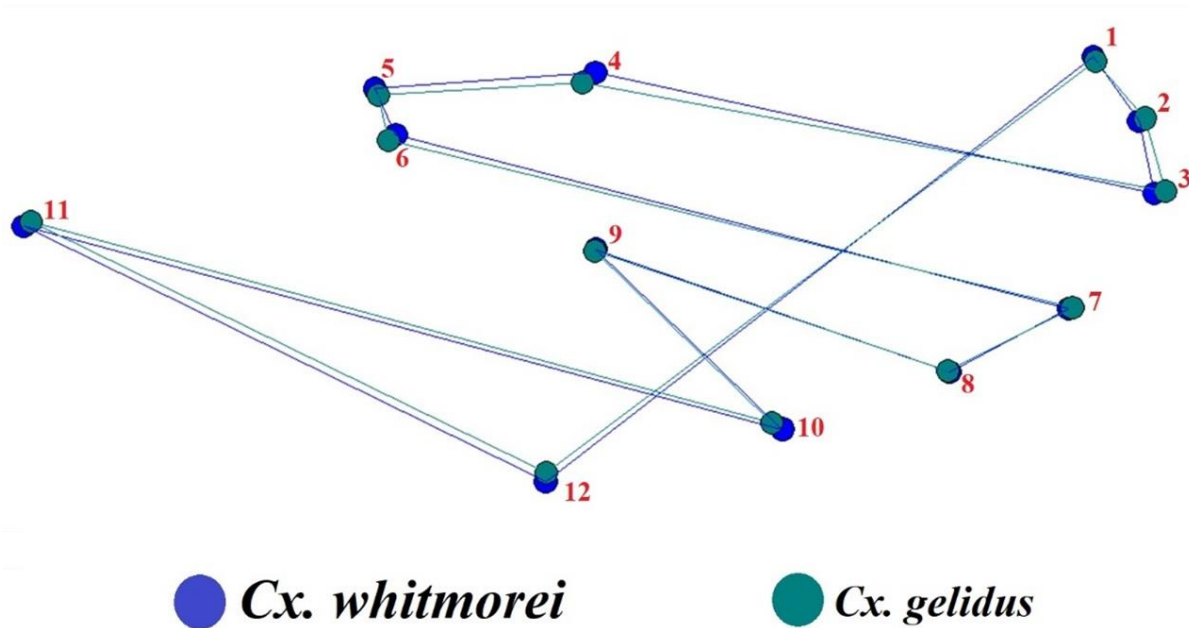| Species	Percent accuracy of assigned individuals	Assigned/observed
Cx. whitmorei	100	25/25
Cx. gelidus	96	24/25

Figure 3. Superimposition of landmark configurations between *Cx. whitmorei* and *Cx. gelidus*.
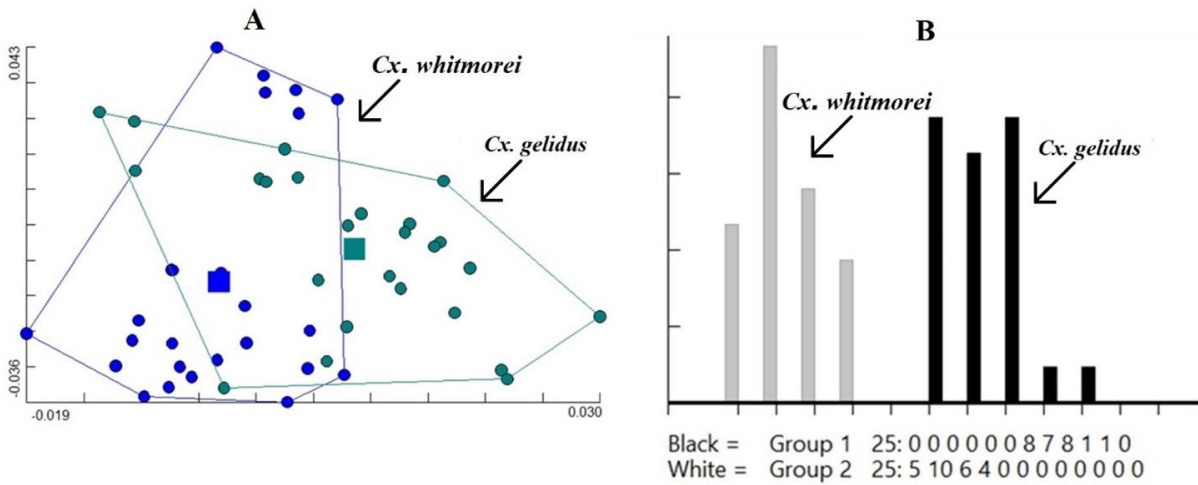

Figure 4. Morphological space (A) and discriminant analysis factor map (B).

DISCUSSION

This study revealed the effectiveness of GM in separating *Cx. whitmorei* and *Cx. gelidus* as vectors of JEV in Thailand. Analysis of interspecific size and shape variation between *Cx. whitmorei* and *Cx. gelidus* demonstrated distinct differences. Mosquito wings were used in the analysis to identify both *Cx. whitmorei* and *Cx. gelidus*, with dimensional structure and venation providing well-defined morphological landmarks.

The results of overall wing size analysis based on CS show that the wings of *Cx. whitmorei* were larger than *Cx. gelidus* and found statistically significant differences between species. Although mean wing size indicates significant differences between species, wing shape is still more important in terms of identification. Mosquito wing size often significantly fluctuates due to environmental influences, such as food availability and larval competition in habitats (Lorenz et al., 2017; Hidalgo et al., 2015). Recently, Demari-

Silva et al., (2014) found wing size differences in *Cx. coronator* in seven areas of Brazil, demonstrating that different environments affect wing size factors of *Culex* mosquitoes. Variations in wing size between coastal and residential areas have also been noted for *Aedes aegypti* (a dengue vector) in different areas of Samut Songkhram Province, Thailand (Chaiphongpachara et al., 2018). Hence, wing size may not be suitable as a target for classification. However, shape is less affected by environmental factors and is primarily determined genetically (Motoki et al., 2012; Lorenz et al., 2012).

For species identification of mosquitoes, wing shape is central to the biological sciences for understanding systematics and evolution (Lorenz et al., 2017). Results of interspecific shape analysis clearly illustrate separation between groups. This result corresponds to the statistical difference of Mahalanobis distance scores based on wing shape among species, consistent with previous research finding that GM methods are successful for separating some species of *Culex* mosquitoes (Wilke et al., 2016). However, some species of *Culex* mosquitoes cannot be adequately identified using GM, requiring a molecular approach instead. Percent accuracy of species identification between *Cx. whitmorei* and *Cx. gelidus* demonstrated high scores (>96%) in cross-validated classification tests. In accordance with previous research, it was found that GM can most effectively identify *Cx. whitmorei* (Chaiphongpachara, 2018). In addition to *Culex* mosquitoes, the method is also highly effective in classifying other mosquito vectors including *Anopheles* species as malaria vectors and *Aedes* species as dengue and Chikungunya vectors.

CONCLUSION

GM is a morphometric approach, successful in identifying many species of mosquitoes with similar morphological characters and is also used to study morphological variations in the environment. The analyses in this study demonstrate the relatively high potential of GM for separating both *Cx. whitmorei* and *Cx. gelidus* as vectors of JEV in Thailand. This success is relevant in terms of separation of both mosquitoes in other areas. It also supports the effectiveness of GM in the identification of mosquito vectors in the field.

CONFLICT OF INTEREST

No conflict of interest

AUTHOR CONTRIBUTIONS

TC designed and wrote the manuscript. SL collected samples and analyzed the data. All authors read and approved the final version.

REFERENCES

Arshad Z, Saxena S, Verma R, Abbas H, Singh GP, Bogra JS, 2013. Japanese encephalitis. A Pi Care 2220-5799. https://doi.org/10.1201/9780203752463.

Bookstein FL, 1991. Morphometric tools for landmark data: geometry and biology. Cambridge. University Press. England pp 55-87.

Chaiphongpachara T, 2018. Comparison of landmark- and outline-based geometric morphometrics for discriminating mosquito vectors in Ratchaburi Province , Thailand. BioMed Res Int https://doi.org/10.1155/2018/6170502.

Chaiphongpachara T, Juijayen N, Chansukh KK, 2018. Wing geometry analysis of *Aedes aegypti* (Diptera,Culicidae), a dengue virus vector , from multiple geographical locations of Samut Songkhram, Thailand. J Arthropod-Borne Dis 12:351–360.

Chaiphongpachara T, Laojun S and Kunphichayadecha C, 2018. Effect of the CDC light trap on control of nocturnal mosquitoes in coastal Samut Songkhram Province , Thailand, Biodiversitas 19: 1750-1754.

Chaiphongpachara T, Laojun S, Kunphichayadecha C, 2019. Effectiveness of ultraviolet (UV) insect light traps for

mosquitoes control in coastal areas of Samut Songkhram province, Thailand. J Anim Behav Biometeorol 7: 25-30.

Demari-Silva B, Suesdek L, Sallum MAM, and Marrelli MT, 2014. Wing geometry of *Culex coronator* (Diptera: Culicidae) from south and southeast Brazil. Parasit Vectors 7: 174.

Dujardin JP, 2008. Morphometrics applied to medical entomology. Infect Genet Evol 8: 875–890.

Hidalgo K, Dujardin JP, Mouline K, Dabiré RK, Renault D, Simard F, 2015. Seasonal variation in wing size and shape between geographic populations of the malaria vector, *Anopheles coluzzii* in Burkina Faso (West Africa). Acta Trop 143: 79–88.

Killick-Kendrick R, 1996. Medical entomology for students. Trans R Soc Trop Med Hyg 20: 1428.

Longbottom J, Browne AJ, Pigott DM, Sinka ME, Golding N, Hay SI, Shearer FM, 2017. Mapping the spatial distribution of the Japanese encephalitis vector, *Culex tritaeniorhynchus* Giles, 1901 (Diptera: Culicidae) within areas of Japanese encephalitis risk. Parasit Vectors https://doi.org/10.1186/s13071-017-2086-8.

Lorenz C, Almeida F, Almeida-Lopes F, Louise C, Pereira SN, Petersen V, Vidal PO, Virginio F, Suesdek L, 2017. Geometric morphometrics in mosquitoes: What has been measured. Infect Genet Evol 54: 205–215.

Lorenz C, Marques T, Sallum MA, and Suesdek L, 2012. Morphometrical diagnosis of the malaria vectors *Anopheles cruzii*, *An. homunculus* and *An. bellator*. Parasit Vectors 5: 257.

Misra UK, Kalita J 2010. Overview: Japanese encephalitis. Prog Neurobiol 91: 108-20.

Motoki MT, Suesdek L, Bergo ES, Sallum MAM 2012. Wing geometry of *Anopheles darlingi* Root (Diptera: Culicidae) in five major Brazilian ecoregions. Infect Genet Evol 12: 1246–1252.

Nemeth N, Bosco-Lauth A, Oesterle P, Kohler D, Bowen R, 2012. North American birds as potential amplifying hosts of Japanese encephalitis virus. Am J Trop Med Hyg 87: 760–767.

Rattanarithikul R, Harbach RE, Harrison BA, Panthusiri P, Jones JW, Coleman RE, 2005. Illustrated keys to the mosquitoes of Thailand. II. Genera *Culex* and *Lutzia*. Southeast Asian J Trop Med Public Health 2:1-97.

Rattanarithikul R, Harrison BA, Pantusiri P and Coleman RE, 2005. Illustrated keys to the mosquitoes of Thailand. Southeast Asian J Trop Med Public Health 1:1-80.

Rohlf FJ, 2002. Geometric morphometrics in systematics. Morphology, Shape and Phylogenetics pp. 175–193.

Wilke ABB, De Oliveira Christe R, Multini LC, Vidal PO, Wilk-Da-silva R, De Carvalho GC, Marrelli MT, 2016. Morphometric wing characters as a tool for mosquito identification. PLos one 11(8).

World Health Organization. 2016. Mosquito born diseases. Retrieved from http://www.who.int/neglected_diseases/vector_ecology/mosquito-borne-diseases/en/. Accessed 28 Decemberber 2018.