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Wing phenotypic variations of the vector *Culex sitiens* in coastal areas of Thailand

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Culex sitiens is a disease vector in coastal areas that transmits important pathogens to humans, including filarial nematodes and Japanese encephalitis virus. Study the variations in size and shape of *Cx. sitiens* mosquitoes in the coastal area of the Samut Songkhram Province using landmark-based geometric morphometric techniques. Mosquitoes were collected from three different coastal areas. Sites were chosen according to their distance from the sea: 200 meters (site A), 2 kilometers (site B), and 4 kilometers (site C). Statistical differences in the wing size for each population were tested using non-parametric permutation with Bonferroni correction test at $\alpha = 0.05$. Shape variation was estimated by Procrustes superimposition (residual coordinates of the 14 anatomical landmarks) following a Generalized Procrustes Analysis and principal components of the partial warps. Significant differences were identified between the wing sizes of populations from site C and those from other sites ($P < 0.05$) and between the wing shapes of the *Cx. sitiens* populations from all sites ($P < 0.05$).Our results provide important information about the microevolutionary patterns, population structure, and phenotypic features of *Cx. sitiens* populations in coastal areas. This information will enhance our understanding of these vectors, leading to more effective vector control.

Keywords: *Culex sitiens*, Coastal areas, Geometric morphometric, Landmark-based

INTRODUCTION

Culex sitiens Wiedemann (Diptera: Culicidae) is a mosquito that lives and distributes in coastal ecosystems (Chaiphongpachara T et al., 2018). Normally, the habitats of *Cx. sitiens* found in coastal areas include ponds, ditches, pits, ground pools, stream margins, salt marshes, crab holes, and mangroves (Rattanarithikul et al., 2005). This species of *Culex* is considered a nocturnal vector, as it transmits important pathogens to humans (Killick-Kendrick, 1996) including the filarial nematode parasite that causes lymphatic filariasis (Rattanarithikul et al., 2005; Iyengar, 1953) and the Japanese encephalitis virus (Vythilingam et al., 1995; Vythilingam et al., 2002). In addition, the high rate of bites caused by *Cx. sitiens* in coastal areas (>100 mosquitoes per person per hour) (Prummongkol et al., 2012) make it a nuisance as well as a health concern. In 2017, the World Health Organization reported that at least 856 million people worldwide live in endemic areas and are at high risk of exposure to lymphatic filariasis (WHO, 2013), and more than 3 billion people are at risk of contracting Japanese encephalitis (Kibler, 2006).

Environment is one of the major factors

affecting mosquito vector populations (Zittra et al., 2017). Coastal areas are often affected by many environmental factors including airflow, water salinity, and rising and falling tides which affect the living organisms in the surrounding area. *Cx*. *sitiens* mosquitoes are often found in coastal areas of Thailand because they breed in high-salinity water (Chaiphongpachara et al., 2018). The salinity of seawater affects numerous living organisms, including some species of mosquitoes, such as incremental populations of brackish-water mosquitoes (Balasubramanian and Nikhil, 2015). In addition, different environments have varying impacts on a vector's ability to transmit diseases (Mouchet and Carnevale, 1997); variability in the environment leads to microevolution, as the mosquito populations respond to environmental influences and adapt to survive (Ramasamy and Surendran, 2012). In addition, phenotypic variations in mosquito populations, including size and shape, are caused by environmental and genetic changes, and these variations may affect vector behavior.

Thus, we The aim of the present study was to investigate the microevolution of *Cx*. *sitiens* in the coastal areas of the Samut Songkhram, Thailand, using a geometric morphometric (GM) technique, which is popular and effective for morphological studies. Samut Songkhram is located on the Gulf of Thailand where *Cx*. *sitiens* are abundant and in which different environments may be identified in each costal area based on their distance from the sea. Our previous research explored the distribution and habitat of mosquito vectors in this coastal area. In these previous studies, we found that the distance from the sea influenced the distribution and diversity of the mosquito species (Chaiphongpachara et al., 2018; Chaiphongpachara and Sumruayphol, 2017). Thus, it is possible that the different coastal area environments may affect phenotypic variations of *Cx*. *sitiens*. Our results provide important information about the micro-evolutionary patterns, population structure, and phenotypic features of *Cx*. *sitiens* populations in coastal areas. This information will enhance our understanding of these vectors, leading to more effective vector control.

MATERIALS AND METHODS

Study areas

Mosquitoes were collected from three different coastal areas. Sites were chosen according to their distance from the sea: 200 meters (site A), 2 kilometers (site B), and 4 kilometers (site C) (Figure 1) following the methods of Chaiphongpachara and Sumruayphol (2017). Site A is characterized by high population density and is semi-urban, and there are both saline water sources and waste water sources distributed in the area (Figure 2a). The mangrove forest in this area is smaller than those of the other sites, as it has been developed as a residential area. Site B is characterized by low population density. There are salt ponds scattered throughout the surrounding area, and there are saline water sources and a mangrove in the area, but these resources are not as plentiful as in site C (Figure 2b). Although site C is located near the sea, the population is lower than in the other sites, and there are mangrove forests and saline water sources distributed throughout the area (Figure 2c).

Mosquito specimen collection

Culex sitiens collections were performed once a week in August 2015 between 18:00 and 06:00 hours using a CDC light trap baited with dry ice. A total of six traps were used for mosquito collections, two traps were used per site. Every morning, we collected the trapped mosquitoes, recorded details, and sent the samples to the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University. Then, *Culex sitiens* samples were identified using the morphological key provided by the Illustrated Keys to the Mosquitoes of Thailand (Rattanarithikul et al., 2010) under a Nikon AZ 100 M stereo-microscope.

Wing preparation

Only the right wing of each female *Cx*. *sitiens* was removed from the thorax and mounted between a glass slide and coverslip using Hoyer's solution. Subsequently, the right wings were photographed using a digital camera connected to a Nikon SMZ745T stereo-microscope under 40× magnification, and the scale bar (1 mm) was added to the picture using NIS-Elements documentation software.

Landmark-based GM approach

Fourteen landmarks at the intersections of wing veins were chosen (Figure 3) and were digitized to Cartesian coordinates.

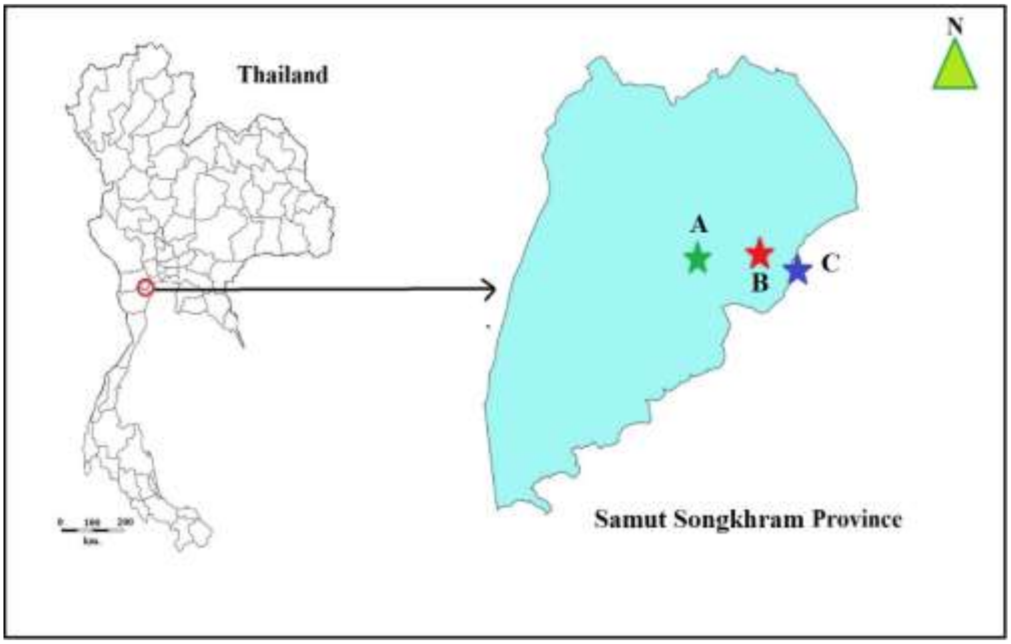

Figure 1; Study areas (green = site A, red = site B, and blue = site C).

Figure 2; General topography of specimen collection sites in each area according to their distance from the sea. a) Site A (4 km away from the sea), left: community distribution in this site, middle: waste water sources, right: saline water sources. b) Site B (2 km away from the sea), left: sea salt farms in this site, middle: mangrove forest, right: saline water sources. c) Site C (200 m away from the sea), left: mangrove forest, middle: sea salt farms, right: located near the sea.

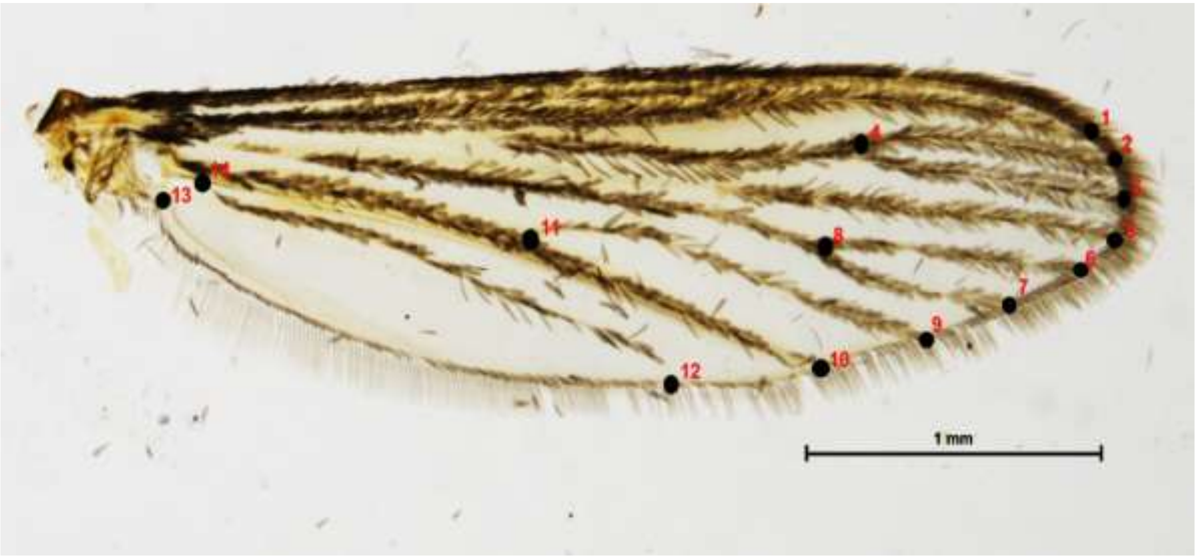

Figure 3; Fourteen landmarks on the right wing of female *Cx. sitiens*.

The wing size of the *Cx*. *sitiens* population from each study site was estimated as the centroid size (CS) derived from the coordinates (Farji-Brener et al., 2015) and size variation at each site was represented by quantile boxes. Then, we evaluated the CS values in each group using non-parametric permutation (1,000 runs) with Bonferroni correction test at α = 0.05.

Shape variation was estimated by Procrustes superimposition (residual coordinates of the 14 anatomical landmarks) following a Generalized Procrustes Analysis and principal components (PCs) (or relative warps [RW]) of the partial warps. After Procrustes superimposition and PCs, discriminant analysis (DA) (or canonical variate analysis**)** derived from the final shape variables was illustrated by the discriminant space map. Shape divergence was estimated by the Mahalanobis distances, which were computed from the DA. A cross-validated classification (or jackknife classification) was used for testing the accuracy of cluster recognition based on wing shape. Each individual sample was reclassified according to its closest group (Mahalanobis distance) without being used to help determine a group center (Manly and Alberto, 2016). The wing shape differences between each specimen from each coastal site was calculated using the difference of Mahalanobis distances derived from shape variables by non-parametric permutation (1,000 runs) with Bonferroni correction tests at α = 0.05.

The *Cx*. *sitiens* population in each coastal habitat and 20 wings of *Cx*. *quinquefasciatus* Say as the out-group (from the Ratchaburi Province) were used to produce the neighbor-joining tree based on Procrustes distances with 1000 bootstrap replicates.

For measurement error of precision in the digitization, data were analyzed by the repeatability index (Arnqvist and Martensson, 1998). Twenty *Culex* images per site were randomly sampled, and the same wing was digitized twice for repeated measurements based on one-way ANOVA (Dujardin, 2011).

Allometry

Environmental changes affect vector size and may produce passive shape changes, which may be explained by allometry (the relationship between size and shape) (Dujardin, 2008). Allometric effects were estimated by multivariate regression of the Procrustes coordinates of the CS and RW, whereas the allometric statistical significance was calculated using non-parametric permutation (1,000 runs) (Good, 1994).

Software

The Collection of Landmarks for Identification and Characterization (CLIC) software package version 97, which is freely available at https://xyom-clic.eu, was used for Landmark-based GM analyses. Various modules of the CLIC program were used in the present study, including the COO module to digitize landmarks; the TET module to modify the data; the MOG module for size and shape analysis; the VAR module to analyze the statistical differences between the CS values and repeatability; and the PAD module to analyze the statistical differences in shape, allometry, and cross-validated classification. Finally, the PHYLIP neighbor module was used to create a neighbor-joining tree.

RESULTS

A total of 122 female *Cx. sitiens* wings were used for the phenotypic variation study using a landmark-based GM approach. All wing samples were obtained from three sites in coastal areas of the Samut Songkhram Province at varying distances from the sea, including 44 wings from site A (4 km), 44 wings from site B (2 km), and 34 wings from site C (200 m).

Repeatability

Comparison of the two replicate sets of digitization data for the same images showed good repeatability scores, and the measurement error was <2% (*Cx. sitiens* from three coastal sites with repeatability indices of 0.985).

Wing size variation

The wing CS variations of *Cx. sitiens* from each coastal site are shown in Figure 4. The wing CS for each area was presented by the mean and standard deviation. The CS of the *Cx. sitiens* from site C was the largest (3.81 ± 0.19 mm), followed by those from sites A (3.64 ± 0.28 mm) and B (3.62 ± 0.21 mm), respectively (Table 1).

Table 1; Statistical analyses of mean wing CS of *Cx. sitiens* in each coastal sites

Site	Mean ± S.D. (mm)	Min–Max (mm)
Site a	3.64 ± 0.28^{a}	2.99–4.07
Site b	3.62 ± 0.21^{a}	3.19–4.11
Site c	3.81 ± 0.19^{b}	3.35–4.09

Different letters indicate statistical differences at $p < 0.05$. Mean, average CS; S.D., standard deviation; Min, minimum; Max, maximum.

The statistical difference between the wing CS values of *Cx. sitiens* from site C was significantly different from those of all sites ($P< 0.05$), whereas sites A and B were not significantly different ($P= 0.77$).

Wing shape variation

The mean landmark configurations of Procrustes superimposition of *Cx. sitiens* from all coastal sites (A, B, and C) are shown in Figure 5. Landmarks 4, 8, and 12 have shown considerable variation in these positions, and it is clear that these landmarks do not completely overlap when data from each site are compared (Figure 5).

The discriminant space map of *Cx. sitiens* from each coastal site by DA showed some overlapping in all sites, but the overlapping was not complete (Figure 6). Similarity comparisons of the wing shapes in each area were evaluated using the Mahalanobis distances values, with sites B and C showing the greatest value (2.31)**.**

The statistical analysis of the Mahalanobis distances of *Cx. sitiens* from each costal site showed that all sites were significantly different ($P < 0.05$) (Table 2).

Table 2; Statistical analyses of Mahalanobis distances of wing shapes of *Cx. sitiens* in each coastal site

	Site a	Site b	Site c
Site a	-		
Site b	1.63*	-	
Site c	1.80*	2.31*	-

* = significant differences at $p < 0.05$**.**

Cross-validated classification scores based on wing shape of *Cx. sitiens* ranged from 40% to 64% and showed high scores in sites C (64%) and B (63%) (Table 3).

Table 3; Cross-validated classification scores based on wing shape of *Cx. sitiens* in each coastal site

Coastal sites	Assigned	n	Percent accuracy of classification
Site a	18	44	40%
Site b	28	44	63%
Site c	22	34	64%

A neighbor-joining tree based on procrustes distances was used to examine morphological variation of *Cx. sitiens* from each coastal site showed that wing phenotypic features are closely related between sites B and A (Figure 7), whereas the outgroup, *Cx. quinquefasciatus*, clearly branched out from the *Cx. sitiens* groups.

Allometric effect

Allometry of shape variables in the present study was free of allometric effects. The differences in wing CS values between localities contributed 12% and were not statistically significant ($P = 2.66$, Figure 8).These values suggest that there was no correlation between the size and shape variables of *Cx. sitiens* populations from each coastal site.

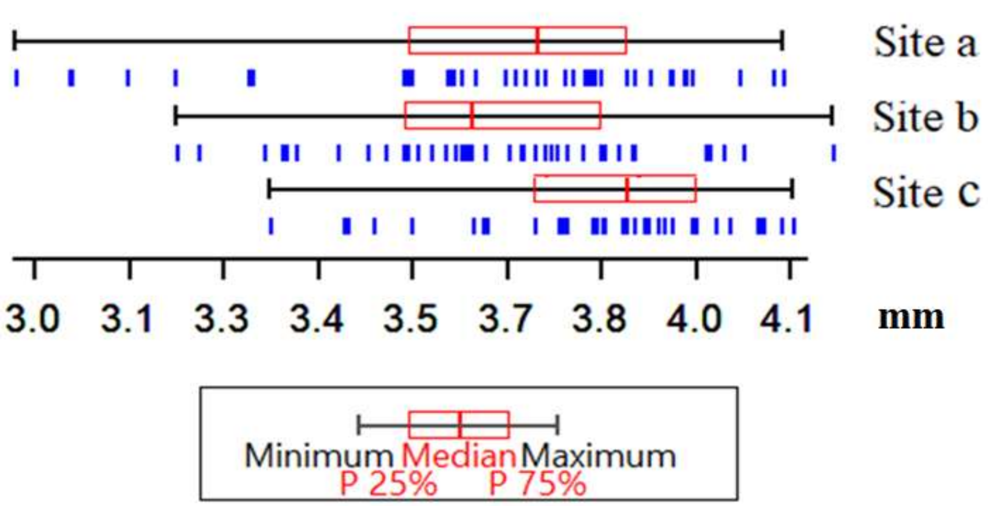

Figure 4; The wing CS (mm) variation of *Cx. sitiens* in each coastal sites was presented by quantile boxes. Each box shows percentiles between 25% and 75% to indicate the median

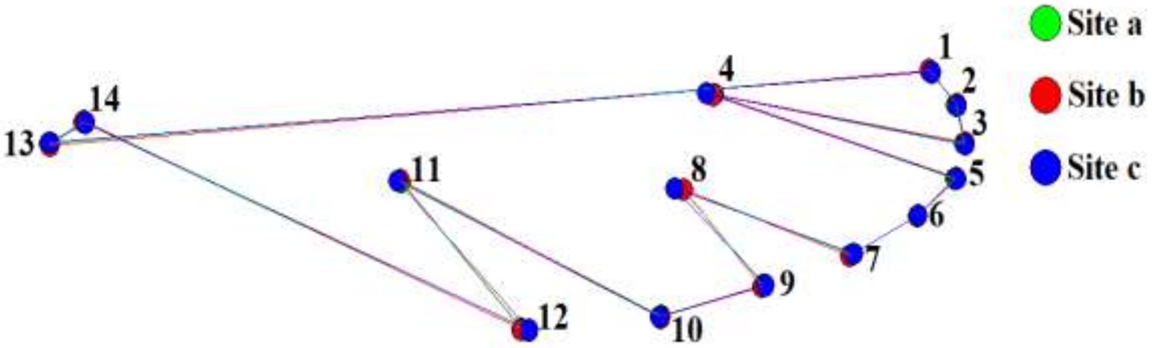

Figure 5; The mean landmark configurations of Procrustes superimposition of *Cx. sitiens* in each coastal site.

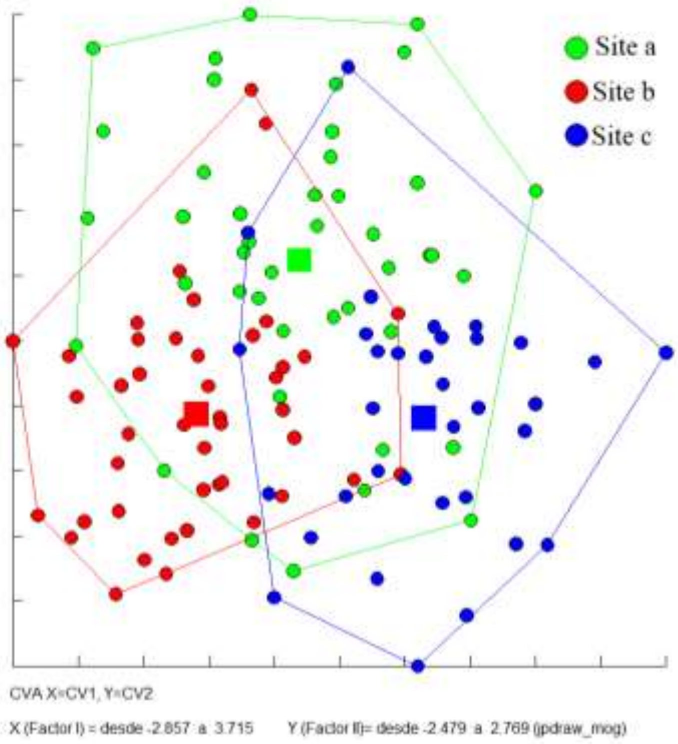

Figure 6; Discriminant space of *Cx. sitiens* in each coastal site based on shape variable by landmark based on DA.

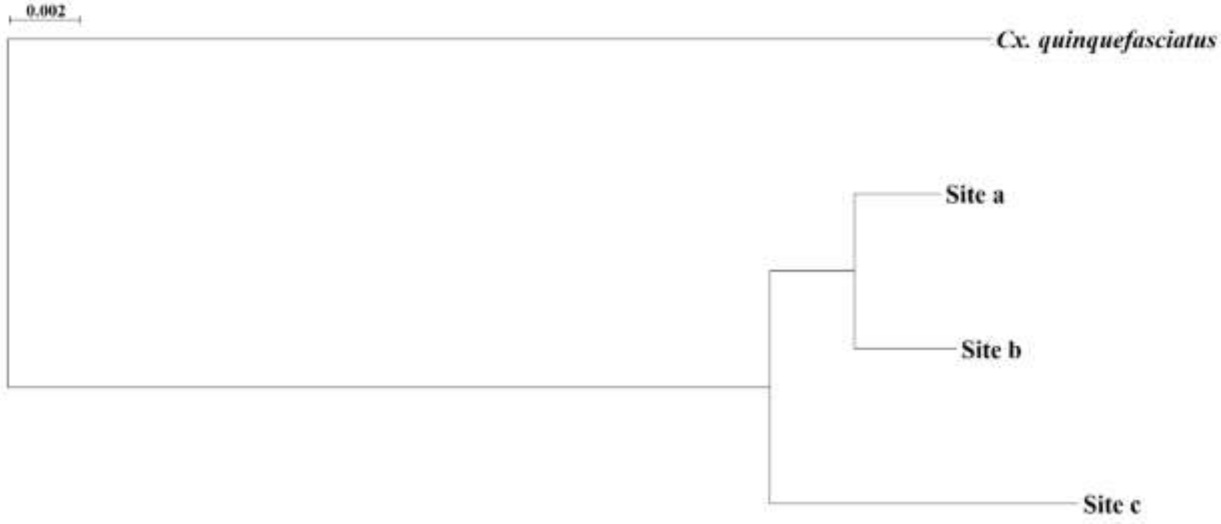

Figure 7; Neighbor-joining tree for *Cx. sitiens* in coastal areas based on wing shape, 1000 bootstrap replicates.

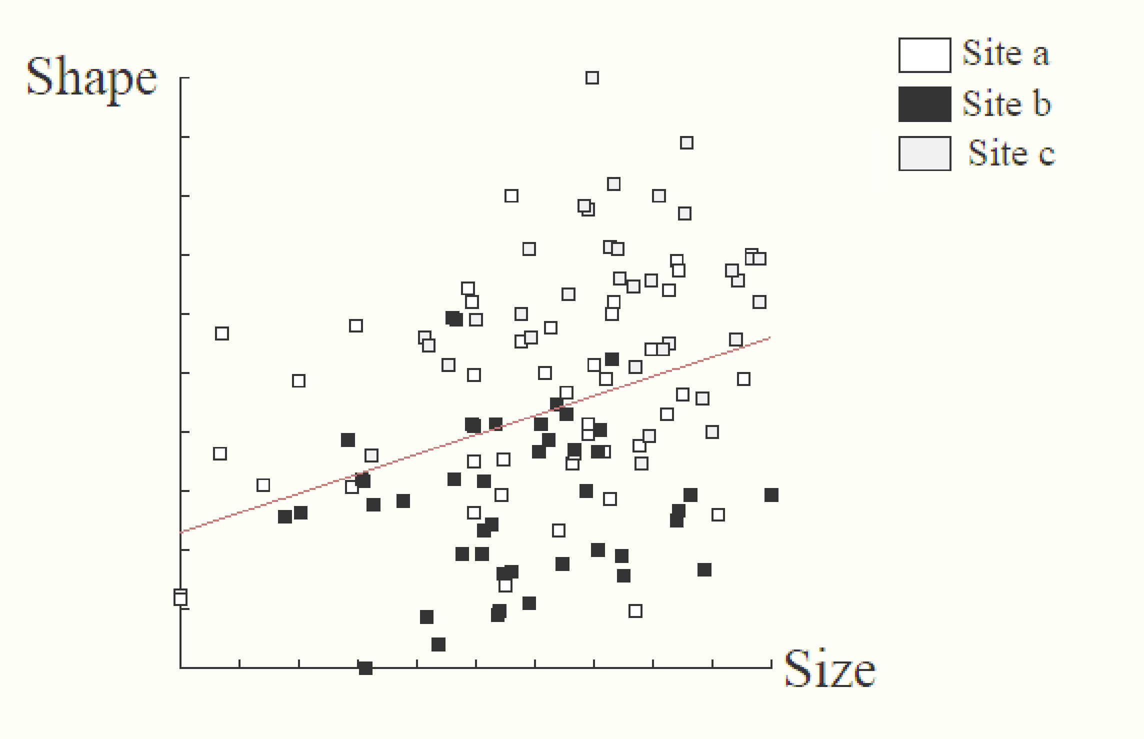

Figure 8; Allometric content of shape variables (RW) illustrated by the influence on shape divergence on size (CS) difference between localities.

DISCUSSION

In the present study, we collected *Cx*. *sitiens* from three coastal areas sites according to their distance from the sea in the Samut Songkhram Thailand, which has an abundance of many coastal mosquito species, including *Cx*. *sitiens* (Chaiphongpachara and Sumruayphol, 2017). *Cx*. *sitiens* is disease vector that plays an important role in coastal areas. A previous study found one *Cx. sitiens* population infected with filarial nematodes in Thailand, but the larvae showed signs of degeneration (Iyengar, 1953). Experiments conducted on the susceptibility of *Cx*. *sitiens* to *Brugia malayi* infection in the laboratory of Prummongkol, Panasoponkul, and Apiwathnasorn (Prummongkol et al., 2009) found that the mosquitoes were resistant to experimental infection (0% of infected mosquitoes). Thus, this species of mosquito may not be highly susceptible to filarial nematode infection. However, reports indicate that it is susceptible to the Japanese encephalitis virus (Vythilingam et al., 1995; Vythilingam et al., 2002).

Coastal areas are environmentally different on the basis of their distance from the sea. A recent research report by Chaiphongpachara and Sumruayphol (2017) surveyed species diversity and habitat in the coastal areas of the Samut Songkhram Province and found differences in species diversity based on their distance from the sea. Three coastal area study sites were used in the present study, including 200 m, 2 km, and 4 km from the sea, and each site had notable environmental differences, such as salinity levels of the available water sources, sea breezes, and mangrove forests.

Analysis of the wing size (CS) of *Cx*. *sitiens* from each coastal area revealed a significant difference between the wing size of specimens from site C and those of other sites ($P < 0.05$). Site C (200 m) is the area closest to the sea, and *Cx*. *sitiens* mosquitoes in this area were the largest in size. Size varies greatly depending on environment; many studies have reported on the size variation of *Culex* mosquitoes in different environments and found that size differences are common. Recently, De Carvalho et al., (2017) have examined wing variations of *Cx*. *nigripalpus* in the urban parks of São Paulo and found many size variations in several parks. The important factor that affects the body size of *Culex* mosquitoes is the quality and quantity of available food (Gordillo and Walton, 2010). This species of mosquito is found predominantly in coastal areas, as it requires access to high-salinity water sources and mangrove forests. Their required resources are typically plentiful in mangrove forests, which are found in site C of this study.

Comparisons between the wing shapes of the *Cx*. *sitiens* population from each coastal area site revealed differences between all sites ($P < 0.05$). These wing phenotypic variations may occur because of the unevenness of the environment (Benítez et al., 2014) in this area. The values of the Mahalanobis distances and cross-validated classification scores report the degree of shape variation. In the present study, the greatest wing phenotypic variation occurs in site C, followed by sites B and A, respectively, corresponding to each site's distance from the sea. Specimens from site C appear to have wings that are different from those from most other sites based on the neighbor-joining tree, which is consistent with the results of wing size variation analysis. Coastal ecosystems are one of the dominant ecosystems that are different from other mainland areas. Apiwathnasorn (2012) reported that insects, including mosquitoes, have the ability to adapt well to changing environments. These results are in agreement with studies of *Culex coronator* wing geometry from seven areas in Brazil that found differences in specimen wing shape in almost half of all areas (10 out of 21 pairs) (Demari-Silva et al*.*, 2014). The impact of the environment on mosquitoes is manifested as changes in the morphology of each species based on genetic expression, as well as changes in vector behavior.

CONCLUSION

Cx. *sitiens* is a major disease vector that spreads diseases to humans and is predominant in coastal areas of many countries. The present study revealed the occurrence of *Cx*. *sitiens* wing phenotypic variations in each coastal area site based on the distance of the site from the sea. Size and shape differences of the *Cx*. *sitiens* populations in coastal areas of Samut Songkhram Province may arise from environmental variations in each site. Our results provide important information about the microevolutionary patterns, population structure, and phenotypic features of *Cx*. *sitiens* populations in coastal areas. This information will enhance our understanding of these vectors, leading to more effective vector control

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

TC designed and performleed the experiments and also wrote the manuscript. SL WW and WSNA performed mosquito collection, and data analysis.

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