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ORIGINAL ARTICLE

Variation over time in wing size and shape of the coastal malaria vector *Anopheles* (*Cellia*) *epiroticus* Linton and Harbach (Diptera: Culicidae) in Samut Songkhram, Thailand

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ABSTRACT

Objective: *Anopheles* (*Cellia*) *epiroticus* Linton & Harbach, a coastal mosquito (also called a brackish mosquito), is a secondary vector species of malaria distributed throughout eastern and southern regions of Thailand. This research aimed to investigate the differences of wing size and shape of this female *Aonpheles* species in Samut Songkhram Province, Thailand occurring over time between 2015 and 2017.
Materials and Methods: Coordinates of 13 landmarks were selected and digitized. Centroid size (CS) was used to estimate wing size. Shape variables were used to estimate wing shape and were calculated from the Generalized Procrustes Analysis following principal components of the partial warp. The statistically significant differences of the average wing size based on CS and wing shape based on Mahalanobis distances in each year were estimated using the non-parametric permutation testing with 1,000 cycles after Bonferroni correction with a significance level of 0.05 ($p < 0.05$).
Results: The *A. epiroticus* population in year 2016 had the highest average (3.61 mm), and the population in year 2017 had the lowest (3.47 mm). In this study, there was no difference in the size of wing between *A. epiroticus* population in the years 2015 and 2016 ($p > 0.05$). The *A. epiroticus* population in year 2017 was significantly smaller than the population in the years 2015 and 2016 ($p < 0.05$). All pairwise comparisons of wing shape Mahalanobis distances were significantly different in year 2017 compared with 2015 and 2016 ($p < 0.01$).
Conclusion: These results indicate differences of wings occur over time that affect the morphological variability of *A. epiroticus*. The differences in weather conditions in each year affect the adaptive and morphological changes of mosquitoes in coastal areas.

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Time variation; wing size; wing shape; *Anopheles epiroticus*; coastal malaria; Samut Songkhram

Introduction

Malaria is the most concerning mosquito-borne disease worldwide. It is found in over 100 countries, and more than 3 billion people at risk of malaria, most of which are in tropical and subtropical climates [1,2]. It is also one of the top three causes of infectious disease illness and death per year, with 247 million cases of malaria worldwide [2]. Malaria is also a major public health concern in the tropical climate of Thailand, particularly along the international borders with three countries, including Cambodia (Northeast and East of Thailand), Myanmar (West and North of Thailand), and Malaysia (South of Thailand) [3,4]. According to the Annual Epidemiological Surveillance Report of Thailand in 2017, there were 2,959 malaria cases and eight deaths throughout the many provinces, especially along international borders [5].

Anopheles mosquitoes act as malaria vectors, and are thus of great medical importance. Globally, there are

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approximately 484 *Anopheles* species but only around 70 act as malaria vectors [6]. In Thailand, there are 74 *Anopheles* species [4] and only around 21 *Anopheles* species are primary and secondary vectors of malaria [7]. *Anopheles* (*Cellia*) *epiroticus* Linton & Harbach, a coastal mosquito (also called a brackish mosquito), is a secondary vector species of malaria distributed throughout eastern and southern regions of Thailand and along the coastal region of Cambodia [8–10]. Recently, Sumruayphol et al. [11] found malaria parasite in *A. epiroticus* in Rayong Province of Thailand [9 positive samples from 926 *A. epiroticus* specimens (six samples infected *Plasmodium falciparum* and three samples infected *P. vivax*)], suggesting that this mosquito should be monitored in coastal areas since it has the potential to transmit malaria. In the past, *A. sundaicus* was considered as a secondary malaria vector which spread throughout the coastal areas of Thailand; however, cytogenetic techniques and DNA methods have now confirmed that *A. sundaicus* species A is one of members in *A.(Cellia) sundaicus* species complex [12], and this species was formally renamed *A. epiroticus* [9,11].

In general, *Anopheles* mosquito population control requires knowledge on biology (life cycle), ecology (habitat), and behavior (feeding and resting behaviors) of each *Anopheles* species to understand the vector potential leading to proper control actions [6], and this knowledge of *A. epiroticus* as a coastal malaria vector in Thailand is still lacking, making it very difficult to reduce the *A. epiroticus* population effectively. Presently, there are several studies examining the morphological variability of mosquitoes, as these changes affect their vector potential [13–15]. It is reported that environmental variations cause changes in the vector dynamics of *Anopheles* mosquitoes, including their reproductive period, disease transmission, and body changes (size and shape) [16–18]. There have been worldwide changes in weather conditions in the recent years, which affects the adaptations and changes in mosquito vector genetics, morphology, and behavior [19]. Each year, there are variations in environmental conditions which may affect mosquitoes in malaria-endemic areas, such as temperature, relative humidity, rainfall, and airstream. In addition, coastal areas have a unique ecosystem, and these areas often receive direct impacts of the environmental and climate changes [20]. Previous research was studied morphological variability of *Culex sitiens* as a Japanese encephalitis vector in coastal areas of Samut Songkhram Province, Thailand and was found that wing sizes and shapes of this *Culex* species were significantly different between observation years [20]. However, it is currently unclear how the changing environmental conditions affect the morphological difference of *A. epiroticus* as a coastal malaria vector in each year.

To gain more information about *A. epiroticus*, we investigated the differences in wing size and shape of coastal *A. epiroticus* in Samut Songkhram Province, Thailand occurring over time between 2015 and 2017. The Samut Songkhram province is a coastal area located on the Gulf of Thailand, and it has been reported that *A. epiroticus* is prevalent in this area [10,21,22]. A landmark-based geometric morphometrics (GM) technique was used to conduct a morphological variation study. This technique has a number of advantages: it is inexpensive, easy to use, and does not require many complex materials or equipment [23–25]. The results of the present study reveal differences in wing size and shape of this species occurring over time between 2015 and 2017, which will lead to more advanced studies further.

Materials and Methods

Anopheles epiroticus mosquito collection and identification

Adult female *A. epiroticus* specimens were collected by CDC light traps (Centers for Disease Control and Prevention) (John W. Hock Co., Gainesville, FL) with dry ice from Mueang Samut Songkhram District of the Samut Songkhram Province in Thailand (13°24’34.2”N, 100°00’53.1”E) once a week during the month of December in 2015–2017 (Fig. 1). The study site was in the coastal area 4 km from the sea, with sources of brackish water scattered throughout the area. Mosquito specimens were collected in December because it is the month after the rainy season in Thailand, during which the area is affected by storms and other environmental conditions, and the *A. epiroticus* mosquitoes are plentiful [11]. However, the weather and the environment vary from year to year. All traps were hung overnight (18:00–06:00 h) at a height of 1.5 m at a 50-m distance from the house. In the morning, mosquito specimens were collected from the traps and sent to the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University. Then, female *A. epiroticus* mosquitoes were identified under a stereo-microscope (Nikon Corp., Tokyo, Japan) based on their physical characteristics using taxonomic keys [26].

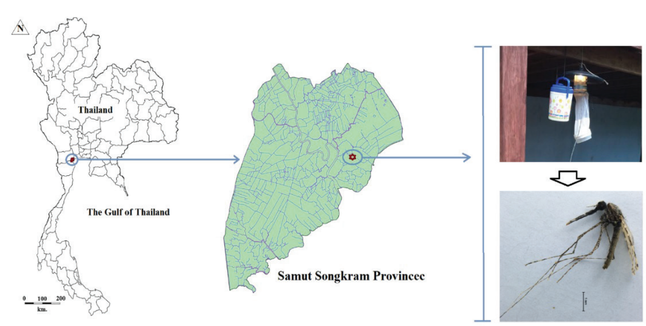

Figure 1. Study site in the Samut Songkhram Province.

Image processing

Anopheles epiroticus wings were used for landmark-based GM analysis in this study. Wings are an ideal organ for GM analysis because they have a nearly two-dimensional organ, making it possible to reduce landmark digitizing errors [27]. The right wing was cut from the body by a needle and mounted on a slide with a cover slip using Hoyer's solution. Then, all the wing samples were photographed using a digital camera connected to a stereo-microscope (40× magnification) with and a 1-mm scale bar was added to all the images.

Collection of coordinates and reproducibility tests for GM analysis

Coordinates of 13 landmarks (LM) were selected and digitized, as shown in Figure 2. The position of these points was chosen based on the clarity of the intersection of the wing lines to prevent visual error. The measurement error of the digitized images was estimated via the repeatability (*R*) index [28]. To ensure the quality and reproducibility of landmark digitization, 20 *A. epiroticus* wing images per each year were randomly selected, and the LM were digitized twice to compare with the dataset used for the analysis. If these results were dissimilar, or one of the groups had a low repeatability index, the LM from each wing were digitized again.

Size variability analysis

The landmark configurations of the specimens collected in each year were translated, scaled, and rotated on the consensus configuration using generalized Procrustes analysis (GPA). Then, centroid size (CS) was used to estimate size of *A. epiroticus* wing, which was defined as the square root of the sum of the squared distances from the centroid to each individual landmark [29,30]. Quantile boxes were created to visualize the variations in CS of *A. epiroticus* in each year.

Shape variability analysis

After GPA (translation, scaling, and rotation) as the procedure of superimposition algorithm, shape variables were used to estimate wing shape and were calculated from the Generalized Procrustes Analysis following principal components of the partial warp [25,29]. Then, discriminant analysis (DA) was used to investigate wing shape dissimilarity in *Anopheles* population in each year. This is shown as a discriminant space map by imputing shape variables, and the Mahalanobis distances scores were calculated from DA to estimate the degree of wing shape similarity in *Anopheles* population in each year. A cross-validated classification test was used to investigate the dissimilarity between consecutive chronological samples based on the Mahalanobis distances [31].

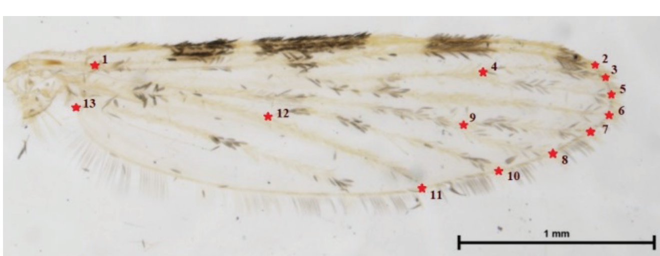

Figure 2. The locations of the 13 LM on the *A. epiroticus* wing (scale bar = 1 mm).

Morphological tree

The pattern of morphological divergence among *A. epiroticus* populations in each year was illustrated by a single-linkage hierarchical classification tree. The bootstrap technique of Couette et al. [32] was used to test the stability of the results via the generation of numerous data sets of the same size by random resampling with the replacement of the variables. After that, in each random data set were analyzed and the bootstrapped trees were calculated. Twenty *A. maculatus* (from Tak Provinces) were added to the classification tree as a putative outgroup.

Data analysis and Software

The statistically significant differences of both wing size based on CS and wing shape based on Mahalanobis distances of *A. epiroticus* populations in each year were estimated using the non-parametric permutation testing with 1,000 cycles after Bonferroni correction with a significance level of 0.05 ($p < 0.05$). XYOM was utilized in the present study, a freely software available at https://xyom.io.

Results

A total of 144 wings of *A. epiroticus* were used to study morphometric variations over time, including size and shape. The samples were divided into 3 years, including 55 wings in 2015, 35 wings in 2016, and 54 wings in 2017. The comparison of two repeated sets of measurements for the same wing images showed good scores. Measurement error was very low (below 1% for size and 3% for shape) indicating repeatability indices of 0.998 in CS estimation and 0.973 in shape (relative warps) estimation.

Size variability

The variation of wing CS between *A. epiroticus* in each year is shown in Figure 3. The *A. epiroticus* population in year 2016 had the highest average (3.61 mm), and the population in year 2017 had the lowest (3.47 mm) (Table 1). In this study, there was no difference in the size of wing between *A. epiroticus* population in the years 2015 and 2016 (Table 1) ($p > 0.05$). The *A. epiroticus* population in

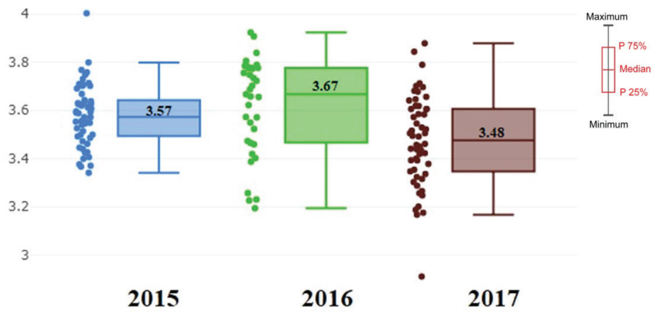

Figure 3. Wing CS variation between *A. epiroticus* populations in each year, shown as quartile boxes. Each box indicates the median scores as a horizontal line that separates the 25th and 75th quartiles.

Table 1. Statistical analyses of mean wing CS of *A. epiroticus* between each year.

Year	*n*	Mean ± SD (mm)	Min–Max (mm)
2015	55	3.58 ± 0.13[1]	2.91–3.87
2016	35	3.61 ± 0.20[1]	3.19–3.92
2017	54	3.47 ± 0.18[2]	3.34–4.00

Different superscript numbers indicate statistical differences at $p < 0.05$.
SD = standard deviation; Min = minimum; Max = maximum.

year 2017 was significantly smaller than the population in the years 2015 and 2016 ($p < 0.05$).

Shape variability

Figure 4 shows the mean landmark configurations of superimposition of *A. epiroticus* populations in each year. DA revealed variations in shape differentiation in each year's population in the discriminant space (Fig. 5). Mahalanobis distances scored as the degree of similarity in wing shape of *An. epiroticus* in each year were found to have the highest value between the populations in 2016 and 2017 (1.96), and the lowest value between 2015 and 2016 (1.18 mm) (Table 2). All pairwise comparisons of wing shape Mahalanobis distances were significantly different in year 2017 compared with 2015 and 2016 ($p < 0.01$; Table 2). The scores of cross-validated reclassification ranged from 40% to 59% based on the Mahalanobis distances, which showed the highest score in year 2017 (59%) followed by 2016 (45%) and 2015 (40%), respectively (Table 3).

Morphological tree

A morphological tree based on single-linkage hierarchical classification revealed that the wing shape variability was closely related between the *A. epiroticus* populations in 2015 and 2016, while the *A. epiroticus* population in year

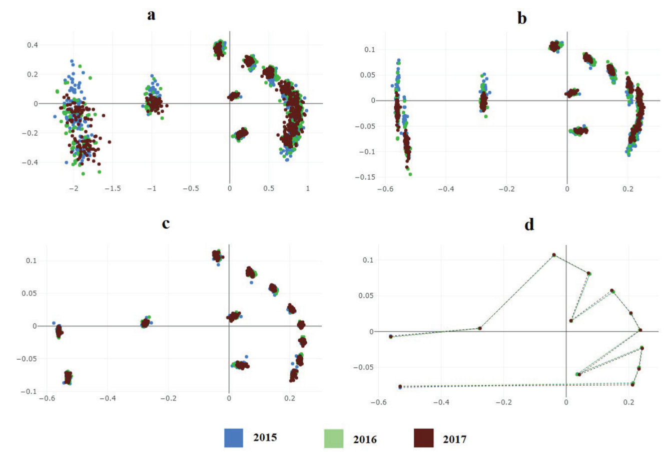

Figure 4. Superimposition of the mean landmark configurations of *A. epiroticus* populations in each year. (d) Before estimated superimposition of the mean landmark configurations, (a) landmark configurations were scaled, (b) translated, and (c) rotated.

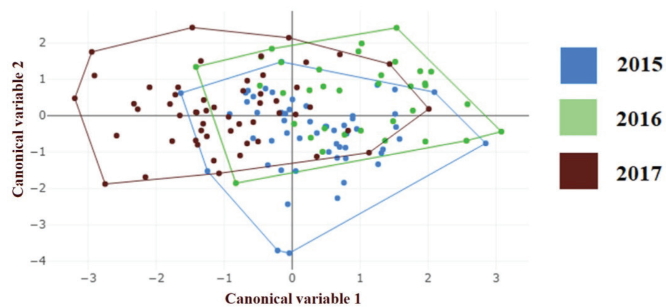

Figure 5. Discriminant space of canonical variables 1 and 2 yielded by DA of wing principal components showing the variation in shape of *A. epiroticus* populations in each year, classified by color.

2017 had a different in shape than in the other years (Fig. 6). *A. maculatus* was used as an outgroup, and was clearly a separate branch from the *A. epiroticus* population.

Discussion

Many environmental factors affect the morphology of mosquitoes, including temperature [33–35], season [36], and geographic area [15,37]. According to the data of annual rainfall and average temperature ranges of Samut Songkram Province in 2015 to 2017 from the Meteorological Department of Thailand, it has reported that rainfall range was different in each year (annual rainfall range: 800–1,000 mm for 2015, 1,000–1,400 mm for 2016, and 1,200–1,600 mm for 2017), while average temperature range was not different (annual average temperature range: 28°C–30°C for 2015–2017) [38–40].

Table 2. Statistical analyses of Mahalanobis distances between wing shapes of *A. epiroticus*.

Years	2015	2016
2015	-	
2016	1.18	-
2017	1.45*	1.96*

* = significant differences at $p < 0.05$.

Table 3. Scores of cross-validated reclassification for *A. epiroticus* populations in each year.

Year	Assig-ned	Observed	Percent (%) accuracy of classification
2015	22	55	40
2016	16	35	45
2017	32	54	59

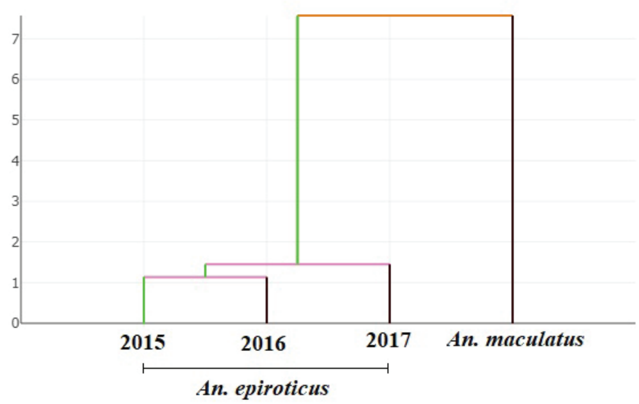

Figure 6. Single-linkage hierarchical classification tree of *A. epiroticus* wing shape in each year, with *A. maculatus* as an outgroup.

Our analysis of the variation in wing size over time revealed differences in size variation between population groups. For wing CS analyses, the *A. epiroticus* population in 2017 was different from that in 2015 and 2016. The effect of the environment on size variation is greater than the effect on the shape. Size variations are reportedly affected during the larval stage by various environmental conditions, such as food availability, nutrition, and larval competition [35,41]. Weather information obtained from the Samut Songkhram Provincial Meteorological Station has revealed a noticeable difference between environmental factors in year 2017 and those in other years: in 2017, the annual average rainfall was above average in Thailand (200–400 mm more than normal criteria [38–40]). This is not consistent with previous reports indicating that rainfall is correlated with larger size of *Aedes albifasciatus* wing in Argentina [42]. The important reason for rainfall affects the small wing size of this mosquito species because *A. epiroticus* is brackish mosquito which has a habitat of larval stage in an appropriate brackish water source [10]. The large amount of rainfall affects the salinity level in the water source, which is the habitat of *A. epiroticus*. The size of mosquito body is one of the factors that influence vectorial capacity [15]; a previous study revealed that the size of *A. arabiensis* affects their longevity, fecundity, and blood meal size, which are related to their ability to transmit pathogens [43].

All pairwise comparisons of the Mahalanobis distances of wing shape were different in year 2017 from those in 2015 and 2016, which is in agreement with the result of the size variability. The single-linkage hierarchical classification tree also indicates a similarity in shape between 2015 and 2016, while in 2017, the wing shape was different from the other 2 years because of the effects of different conditions. This is in agreement with the results of the study published by Gomez et al. [17], which reported that rainfall was factor that influenced the shape variation in *A. albimanus* as a major malaria mosquito vector in Colombia. Presently, there are many studies examining the morphological variation of *Anopheles* mosquitoes in different geographic areas reporting variations in population size and shape [17,18]. Different environmental factors cause morphological variations in mosquitoes, and these factors influence the differences of wing size and shape over time. In addition, the results in this study were consistent with the previous research that found differences in the wing morphology, including wing size and shape of *C. sitiens* in Thailand between annual populations [20]. Currently, worldwide weather conditions are fluctuating in each year, which also affects the adaptation and morphological changes of mosquitoes [19,44], and thus affects their ability to transmit the disease to humans [10].

Conclusion

This study revealed that morphological variations occur in the costal malaria vector *A. epiroticus* over time, including changes in wing size and shape. The differences of weather conditions in each year affect the difference in morphology of mosquito populations. Our results clearly indicate that differences in the size and shape of *A. epiroticus* mosquitoes were observed during years in which variations in rainfall patterns occurred. GM is one of effective techniques commonly used to study the variation in vectors, which has the advantage of being inexpensive, rapid, and easy to use. Based on the results, to avoid the effects of variations in wing size and shape of mosquito that may occur over time, we recommend using specimens collected in the same year for the study of morphological variation.

Acknowledgment

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Conflict of Interests

The authors declare that they have no conflict of interest.

Authors' contribution

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

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