Research Article

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Wing morphometric variability of the malaria vector *Anopheles (Cellia) epiroticus* Linton et Harbach (Diptera: Culicidae) for the duration of the rainy season in coastal areas of Samut Songkhram, Thailand

Tanawat Chaiphongpachara¹, Sedthapong Laojun²

¹ Department of Public Health and Health Promotion, College of Allied Health Science, Suan Sunandha Rajabhat University, Samut Songkhram, Thailand.

² College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, Thailand.

Abstract: In Thailand, *Anopheles (Cellia) epiroticus* Linton et Harbach (Diptera: Culicidae) is the secondary vector of human malaria along coastal regions. While there are some studies of phenotypic variability and population structure of *A. epiroticus*, more information on morphological variation would enhance epidemiological understanding of medically important mosquito vectors. This research examined morphological variation at three different distances from coastlines of Samut Songkhram Province, Thailand, using landmark-based geometric morphometrics. Wing shape of *A. epiroticus* was significantly different in the area 0.2 km away from the sea compared to areas 2 and 4 km away from the sea ($p < 0.05$). Phenotypic variability in wing shape is associated with distance from the sea. Morphological variations in the area closest to the sea were most pronounced, showing a relationship between *A. epiroticus* and the ecosystem that affects wing geometry. These results provide important information to understand morphological variation of *A. epiroticus* in coastal areas.

Keywords: Mosquito, microevolution, coastal environment, morphological variability, geometric morphometrics.

Mosquito-borne diseases cause health problems for humans worldwide, especially in tropical and subtropical countries which are recognised as areas of abundance for several mosquito vector species (Tolle 2009). Malaria, the most important mosquito-borne disease, is one of the top global causes of infection and death (Gómez et al. 2014). Globally, an estimated 219 million malaria cases were reported by the World Malaria Report 2017 (World Health Organisation 2017). The mosquito *Anopheles epiroticus* Linton et Harbach, belonging to the family Culicidae (Diptera), is a major vector of malarial *Plasmodium* in coastal areas of many countries (Linton et al. 2005), including Thailand (Sumruayphol et al. 2010).

In Thailand, malaria causes significant morbidity which is ongoing in remote hilly and forest areas, especially along the western border areas with Myanmar and the eastern border areas with Cambodia, far from health facilities (Ritthison et al. 2014). Some of these cases occurred in coastal areas, the habitat of *A. epiroticus*, the secondary malaria vector in Thailand. *Anopheles epiroticus* can transmit the malaria parasite in coastal areas. For example, a study by Sumruayphol et al. (2010) in Rayong Province (a coastal province) found

0.97% of *A. epiroticus* infected with *Plasmodium falciparum* Welch, 1897, and *P. vivax* Grassi et Feletti, 1890.

Recently, new details about biology of *A. epiroticus* have come to light, including information on behaviour (Sumruayphol et al. 2010, Ritthison et al. 2014, Chaiphongpachara et al. 2018) and the species complex (Linton et al. 2005). Environmental impact on mosquito adaptations is an important factor in understanding this vector. The natural environment affects factors such as growth, biting behaviour, species diversity, distribution, morphology and genetics (Apiwathnasorn 2012).

During the last decade, mosquito vectors' morphological variability across different locations has been studied, such as *Aedes* Meigen as dengue vectors (Henry et al. 2010) and chikungunya vectors (Morales et al. 2013), *Culex* Linnaeus as encephalitis vectors (Demari-Silva et al. 2014, De Carvalho et al. 2017), and *Anopheles* Meigen as malaria vectors (Vicente et al. 2011, Motoki et al. 2012, Lorenz et al. 2014, Hidalgo et al. 2015). More information on morphological variation can enhance epidemiological understanding of medically important mosquito vectors (Dujardin 2011).

Address for correspondence: Tanawat Chaiphongpachara, Department of Public Health and Health Promotion, College of Allied Health Science, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand. Phone: 083-5865775. E-mail: tanawat.ch@ssru.ac.th

Fig. 1. Location of the three coastal study sites (Green indicator: site 4 km from the sea, Red indicator: site 2 km from the sea, Blue indicator: site 0.2 km from the sea).

Fig. 2. Thirteen landmarks on an wing of *Anopheles epiroticus* Linton et Harbach.

Some researchers have reported a relationship between size and vector competence which shows larger mosquitoes support a greater number of parasites (Pulkkinen and Ebert 2004, Moller-Jacobs et al. 2014). In addition, larger mosquitoes have longer lifespans, which increases the likelihood of their transmitting pathogens to humans (Ameneshewa and Service 1996). However, data on phenotypic variability and population structure of *A. epiroticus* are still lacking.

Samut Songkhram, a seaside province, is located on the Gulf of Thailand, central Thailand. Around half of the province is coastal with abundant natural resources, and

different ecology depending on the distance from the sea (Chaiphongpachara and Sumruayphol 2017, Chaiphongpachara et al. 2017). This area is a confirmed habitat for *A. epiroticus*, and its distribution varies according to distance from the sea (Chaiphongpachara and Sumruayphol 2017). It is possible, then, that these mosquitoes exhibit morphological variation depending on environmental differences and the ecosystems of different coastal areas.

This research focused on variation in wing morphology of *A. epiroticus*, including size and shape, across the duration of the rainy season. Coastal areas were classified by distance from the coastlines, including 0.2 km, 2 km and

Fig. 3. Variation in wing centroid size (mm) between *Anopheles epiroticus* Linton et Harbach populations at each distance from the sea including 4 km, 2 km and 0.2 km, shown as quartile boxes. Each box indicates the median position as a line across the middle separating the 25th and 75th quartiles.

4 km in Samut Songkhram, Thailand. Geometric morphometrics (GM), a simple, low-cost method was used in this study as it does not require any highly specialised tools other than standard laboratory equipment such as a stereo microscope (Rohlf 2002).

MATERIALS AND METHODS

Study sites

This study was conducted in the coastal Samut Songkhram Province of Thailand, which is located at the Mae Klong River estuary. Based on different environments, three coastal sites were selected as study sites, including 0.2 km (13°24'01.8"N; 100°02'37.5"E), 2 km (13°25'11.7"N; 100°02'21.0"E) and 4 km away from the sea (13°24'56.4"N; 100°00'25.88"E), based on previous research by Chaiphongpachara and Sumruayphol (2017) which was conducted to survey the biology of this mosquito species on the Samut Songkhram coast (Fig. 1.)

Collection and identification of *Anopheles epiroticus*

Adult mosquitoes were collected at the three coastal sites once a week throughout August 2015 from 6.00 pm to 6.00 am using Centers for Disease Control and Prevention miniature light traps (CDC-LT) (John W. Hock Co., Gainesville, Florida) baited with dry ice. Six CDC-LTs (two traps per site) were used, hung about 1.5 m high, and located 50 m from the nearest dwelling. At 6.00 am, the mosquito samples were collected from the CDC-LTs, their details recorded, and samples carefully transported to the biology laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Provincial Education Centre. Subsequently, female *Anopheles epiroticus* were identified under a stereomicroscope (Nikon Corp., Tokyo, Japan) using a morphological key (Rattanakrithikul et al. 2010).

Preparation of wings for Geometric morphometrics

The right wings of female mosquito samples were exquisitely dissected from the thorax part by a teasing needle and fixed on a glass microscope slide (size 25 mm × 75 mm) with a cover slip

by Hoyer's mounting medium (called gum chloral) which contains: 50 ml distilled water, 30 g gum arabic [USP flake], 200 g chloral hydrate, and 20 ml glycerol). A digital camera and photographic equipment connected to a stereomicroscope (Nikon Corp., Tokyo, Japan: fitted with plain lenses to minimise image distortion) under 40× magnification was used to photograph wing images. A 1 mm scale bar was embedded into all images using the NIS-Elements documentation program.

Landmark-based Geometric morphometrics approach

Landmark collection and repeatability

Thirteen unambiguous landmarks (at wing vein intersections) were digitised from the wing images (Fig. 2). In this study, measurement error was investigated and calculated using the repeatability index (Arnqvist and Mårtensson 1998) by comparing two digitised image sets for precision checking. Twenty specimens were chosen at random from each coastal site and digitised again for iterative calculations based on an ANOVA design (Dujardin 2011).

Wing size

The landmark configurations of each sample group were translated, scaled, rotated on the consensus configuration and analysed orthogonal projection (ORP) of aligned samples (Generalised Procrustes Analysis), and the size was calculated. Centroid size (CS) was used as the size measure of GM, defined as 'the square root of the sum of the squared distances from the centroid to each landmark' (Rohlf and Slice 1990, Bookstein 1991), to estimate wing size of *A. epiroticus*. The variability in wing CS (in mm) of samples from each coastal site was presented by quartile boxes between 25% and 75%. Statistical comparison of differences in average wing CS between each site was performed using a non-parametric permutation (10,000 runs) and a Bonferroni correction test, indicating significance at a p-value less than 0.05.

Wing shape

After Generalised Procrustes Analysis (GPA or Procrustes superimposition) as the method of superimposition to compute

Fig. 4. Superimposition of the mean landmark configurations of *Anopheles epiroticus* Linton et Harbach populations at each distance from the sea including 4 km (green), 2 km (red) and 0.2 km (blue).

shape coordinates, wing shape variables (or partial warps PW) were computed by Principal Component Analyses (or relative warps – RW) of the PW scores (Rohlf and Slice 1990, Dujardin 2011). Discriminant analysis (DA or Canonical Variate Analysis CVA) as a method for discriminating biological groups was used to illustrate the discriminant feature spaces by input variables using the RW. Mahalanobis distances as a statistical measure to quantify the distance between group centroids, were computed from DA to assess the similarity between mosquito populations. The statistical significance of shape differences, based on pairwise Mahalanobis distances between populations in each coastal site, was analysed using a non-parametric permutation (10,000 runs) after a Bonferroni correction test, indicating significance at a p -value less than 0.05.

A cross-validated classification (jack-knife classification) was used to confirm the shape variability and to test the accuracy of cluster recognition based on wing shape of the population in each coastal site. Each sample was reclassified according to wing shape similarity of each group of *A. epiroticus*.

Neighbour-joining tree

A neighbour-joining tree, a structure in which groups of *Anopheles* in each site are arranged on branches, was generated, based on Procrustes distances, to illustrate morphological divergence among *A. epiroticus* populations. Twenty right wings of *Anopheles maculatus* Theobald, 1901, the primary malaria vector in Thailand, were taken as an outgroup (from Tak Province, western Thailand).

Allometric analysis

Environmental conditions and other factors in the ecosystem can affect an organism's size and may produce passive shape changes such as some insect vectors (Dujardin 2008). Normally, size and shape variables are not independent attributes, and shape is not an allometry-free variable (Dujardin 2011). For analysis, allometry, the relationship between size and shape, was estimated by multivariate regression of the Procrustes coordinates using wing size (CS) and wing shape (RW).

Software

Landmark-based GM analysis was performed using the various modules of CLIC software (Collection of Landmarks for Identification and Characterisation) (Dujardin et al. 2010), available free at <https://xyom-clic.eu>. Five modules of CLIC were applied

for GM analysis, including the COO (the Collection of Coordinates) module to digitise 13 landmarks on wing vein intersections; the TET (the Tabla, Espacios, Texto) module to convert or compute data; the MOG (the Morfometria Geometrica) module to analyse wing CS and shape variables including GPA, PW, RW and DA; the VAR (the Variation and variance) module to analyse statistical CS differences and the repeatability test; the PAD (the Permutaciones Analisis Discriminante) module to analyse statistical differences in wing shape (based on Mahalanobis distances); the allometry test, and cross-validated classification. Finally, the PHYLIP neighbour module and NJPLOT software (Perrière and Gouy 1996), available free at <http://doua.prabi.fr/software/njplot>, were used to compute and create a neighbour-joining tree.

RESULTS

In this study, 143 wings of *Anopheles epiroticus* were used for morphometric variability analysis using the landmark-based GM approach. All samples were collected from the three study sites in coastal areas of Samut Songkhram Province: 54 wings from the site 4 km from the sea, 55 wings from the site 2 km from the sea, and 34 wings from the site 0.2 km from the sea. The precision in the digitisation of 13 anatomical landmarks, measured in duplicate, showed good scores, giving a very low error measurement (< 1%) and a repeatability index of 0.992.

Wing size variability

The CS variation between populations at each coastal site is shown in Fig. 3. CS ranged from 2.85 to 4.02 mm (Table 1). The mean CS was highest at the sites 4 km and 0.2 km from the sea with a value of 3.47 mm, and lowest at the site 2 km from the sea with a value of 3.37 mm (Table 1). The mean wing CS at all sites did not differ significantly ($p > 0.05$) based on a non-parametric permutation and the Bonferroni correction test.

Wing size variability

The mean landmark configurations of GPA of populations of *A. epiroticus* at each coastal site are shown in Fig. 4, while shape variation, as shown by discriminant space, is illustrated in Fig. 5.

The Mahalanobis distance was highest between the sites 0.2 km and 4 km away from the sea with a value of 1.86, and lowest between sites 2 km and 4 km away from the sea

Fig. 5. Discriminant space of DA (CVA) showing the morphological variation in *Anopheles epiroticus* Linton et Harbach wing shape among populations in each distance from the sea, classified by colour including 4 km (green), 2 km (red) and 0.2 km (blue).

with a value of 1.23 (Table 2). The Mahalanobis distances of wing shape by non-parametric tests were significantly different between the sites 0.2 km and 2 km from the sea, and between the sites 0.2 km and 4 km from the sea (Table 2). The cross-validated reclassification score was highest at the site 0.2 km from the sea (67%), followed by that at 4 km from the sea (46%), and lowest at the site 2 km from the sea (38%) (Table 3).

Allometric effects

Shape variables in this study were not free from allometric effects. The relationship (by linear regression) between wing CS did not influence shape divergence. Wing CS differences between localities contributed a 14% influence to variation in wing shape and were not statistically significant ($p = 1.28$).

Neighbour-joining tree

A neighbour-joining tree was used to examine morphological variation of *A. epiroticus* at each coastal site. Phenotypic variability in wing shape indicated the closest relationship between the populations 2 km and 4 km from the sea, and showed a different shape at the site 0.2 km from the sea (Fig. 6). The *Anopheles maculatus* outgroup branch was clearly separated from those of *A. epiroticus*.

DISCUSSION

This research studied morphometric variability of wings of *Anopheles epiroticus* in coastal areas of Samut Songkhram Province, Thailand, classified by different distances from the sea. This mosquito is commonly found near the coast and is usually associated with brackish water or saline water ecosystems (Linton et al. 2005). In Thailand, *A. epiroticus* is common in coastal areas on the Gulf of Thailand and the Andaman Sea (Tainchum et al. 2015), and was proven a secondary vector of malaria in Thailand (Sumruayphol et al. 2010). In 2010, Sumruayphol et al.

Table 1. Statistical analysis of mean size of wing of *Anopheles epiroticus* Linton et Harbach based on CS.

Coastal sites	n	Mean $\pm$ S.D. (mm)	Min–Max (mm)
Distance from the sea 4 km	54	3.47 $\pm$ 0.06 ^a	2.91–3.88
Distance from the sea 2 km	55	3.37 $\pm$ 0.06 ^a	2.85–4.02
Distance from the sea 0.2 km	34	3.47 $\pm$ 0.05 ^a	3.13–3.71

Different superscript lowercase letters indicate a significant difference at $p < 0.05$. S.D. – standard deviation; Min – minimum; Max – maximum.

Table 2. Statistical analysis of wing shape based on Mahalanobis distances.

Coastal sites	4 kilometres	2 kilometres	0.2 kilometres
4 kilometres	-	-	-
2 kilometres	1.23	-	-
0.2 kilometres	1.86*	1.71*	-

* = significant difference at $p < 0.05$. Distances equate to site distances from the sea.

(2010) reported the presence of *Plasmodium falciparum* and *P. vivax* in 9 of 926 (0.97%) *A. epiroticus* mosquitoes tested in Rayong Province, Thailand. It has recently been reported that in small coastal areas where ecosystems differ according to their distance from the sea of diversity and distribution, mosquito vectors may be affected, exhibiting morphological variation (Chaiphongpachara and Sumruayphol 2017). Following reports demonstrating the usefulness of a landmark-based GM approach to assessing morphological variability of *Anopheles* spp. mosquitoes in many countries around the world (Vicente et al. 2011, Motoki et al. 2012, Gómez et al. 2014, Lorenz et al. 2014, Hidalgo et al. 2015), such an approach was used in this study.

In this work, we were confronted with data showing no statistically significant difference of wing CS in all sites. The size of adult mosquitoes is a result of the environment at breeding sites during the immature stage in water, such as food availability, nutrition and larval competition (Gómez et al. 2014, Moller-Jacobs et al. 2014, Shapiro et al. 2016). These factors are positively associated with a larval diet which affects the size of adults (Gómez et al. 2014, Moller-Jacobs et al. 2014, Shapiro et al. 2016). However, previous research on the distribution of *A. epiroticus* in coastal habitats of Samut Songkhram Province reported that three larval habitats away from the sea have similar biological factors (Chaiphongpachara and Sumruayphol 2017) which make no difference in the wing size of mosquitoes in this study.

Wing shape analysis (based on Mahalanobis distance) in coastal areas located at differing distances from the sea revealed a shape difference in the site closest to the sea (0.2 km) compared to the other areas. The neighbour-joining tree showed that the mosquito population in the site 0.2 km from the sea differs morphologically from the populations in the other areas. The environmental variation is wide in the coastal area, often most prominently in the areas closest to the sea. Salinity levels, the presence of mangrove forests and air streams are different in each part of the coastal area and increase nearer the sea (Chaiphongpachara and Sumruayphol 2017). While mosquito popu-

Fig. 6. A neighbour-joining tree based on wing shape of *Anopheles epiroticus* Linton et Harbach with 10,000 bootstrap replicates (Distances equate to site distances from the sea).

Table 3. Cross-validated reclassification scores based on wing shape similarity.

Coastal sites	Assigned/Observed (individual)	Percent (%) accuracy of classification
Distance from the sea 4 km	25/54	46
Distance from the sea 2 km	21/55	38
Distance from the sea 0.2 km	23/34	67

lations 2 and 4 km from the sea in this study did not show differences, both sites differ in ecology, probably because the factors that affect *A. epiroticus* are the influence of the unique ecosystem of the coastal area.

Recently, using a global climate change model, Garner et al. (2015) reported that sea-level and climate change affect coastal plant species. Sumruayphol et al. (2017) compared the variability of the two main sandflies (vectors of leishmaniasis) in Thailand between island and mainland locations. They found differences in wing shape, with greater morphological variations in the island environment. Areas with contrasting ecosystems have variable mosquito populations (Vicente et al. 2011, Motoki et al. 2012). Motoki et al. (2012) found shape variation in *Anopheles darlingi* in five Brazilian eco regions with very different environmental conditions.

The differences in shape of *A. epiroticus* according to the distance from the sea in this study may be caused by the environmental impact on the morphology. Surveys of annual variability of wing size and shape of two coastal mosquito vectors including *A. epiroticus* and *Culex sitiens* Wiedemann in the coastal areas of Samut Songkhram from 2015 to 2017 indicated that although the mosquito population is in the same ecosystem, other factors may affect the morphological variability of the population (Chaiphongpachara and Laojun 2019a,b).

This study is the first to reveal the morphological variability of the malaria vector *A. epiroticus* in the coastal ecosystems of Thailand. This phenotypic variation in shape is associated with distance from the sea. Morphological variations of *A. epiroticus* across the duration of the rainy season are most pronounced in the area closest to the sea. These results provide important information for understanding the morphological variation of this secondary malaria vector in Thailand, and may ultimately lead to more advanced studies of the biology of this species.

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