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RESEARCH

Insights into the mitochondrial cytochrome oxidase I (mt-*COI*) gene and wing morphometrics of *Anopheles baimaii* (Diptera: Culicidae) in malaria-endemic islands of Thailand

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Abstract

Anopheles baimaii (Diptera: Culicidae) significantly contributes to the transmission of parasites causing malaria in Southeast Asia and South Asia. This study examined the morphological (wing shape) and molecular (mitochondrial gene) variations of *An. baimaii* in four of Thailand's border islands, and also investigated the presence of *Plasmodium* parasites in these mosquitoes. No *Plasmodium* infections were detected in the samples. Significant differences in wing shape were observed in most island populations ($p < 0.05$). A single-linkage tree, constructed using Mahalanobis distances, clustered the populations into two groups based on geographical locations. Genetic variation in *An. baimaii* was also analyzed through cytochrome *c* oxidase subunit I (*COI*) gene sequences. This analysis identified 22 segregating sites and a low nucleotide diversity of 0.004. Furthermore, 18 distinct haplotypes were identified, indicating a high haplotype diversity of 0.825. Neutrality tests for the overall population revealed a significantly negative Fu's *Fs* value (-5.029), indicating a population expansion. In contrast, Tajima's *D* yielded a negative value (-1.028) that did not reach statistical significance. The mismatch distribution analysis exhibited a bimodal pattern, and the raggedness index was 0.068, showing no significant discrepancy ($p = 0.485$) between observed and expected distributions. Pairwise genetic differentiation assessments demonstrated significant differences between all populations ($p < 0.05$). These findings provide valuable insights into the *COI* gene and wing morphometric variations in *An. baimaii* across Thailand's islands, offering critical information for understanding the adaptations of this malaria vector and guiding future comprehensive research.

Keywords Geometric morphometrics · Genetic variation · *Anopheles baimaii* · Mitochondrial cytochrome oxidase I · Malaria · Islands in Thailand

Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites—unicellular protozoans in the Apicomplexa phylum—and is transmitted through infected female *Anopheles* mosquitoes (Cox 2010). This disease is most prevalent in tropical and subtropical regions, particularly in Africa (World Health Organization 2023). Despite extensive control and elimination efforts, malaria continues to pose a significant public health challenge. Recent World Health Organization data indicate a worrying rise in global malaria cases, increasing to approximately 247 million in 2021 from 245 million in 2020 and 232 million in 2019 (World Health Organization 2023). This escalating trend underscores the ongoing difficulties in combating malaria worldwide.

The *Anopheles* genus of mosquitoes plays a crucial role in public health as a malaria vector. Comprising over 500

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species (Harbach 2023), approximately 30–40 of these are known vectors of *Plasmodium*, the parasite causing malaria in humans (Fikadu and Ashenafi 2023). *Anopheles baimaii* Sallum & Peyton, 2005, formerly known as *Anopheles* (*Cellia*) *dirus* species D, significantly contributes to the transmission of the malaria *Plasmodium* parasites in Southeast Asia and South Asia (Obsomer et al. 2007; O'Loughlin et al. 2008). This species is found from the northeastern regions of India, through the hills of Bangladesh and Myanmar, to northwestern and southern Thailand (Obsomer et al. 2007). *Anopheles baimaii* typically resides in dense forest areas and breeds in stagnant water bodies located in shaded areas. Common breeding sites for this mosquito species include pot-holes, ground pools, depressions in forest areas characterized by fallen leaves, animal footprints, tire tracks, and other small bodies of water (Sarmah et al. 2019; Khan et al. 2023).

Thailand, located in the tropical region of Southeast Asia, faces ongoing challenges with malaria as a significant public health issue (Ministry of Public Health of Thailand 2023). Although the country has made strides in reducing malaria incidence overall, persistent transmission remains, particularly in forested border areas adjacent to Myanmar, Cambodia, and Malaysia (Thimasarn et al. 1995; Parker et al. 2015). The intimate connection between local communities and these forests complicates malaria control (Fungladda and Butraporn 1992). Additionally, Thailand's archipelago, a draw for both domestic and international tourists due to its natural beauty, is also a hotspot for malaria, transmitted primarily by *Anopheles* mosquitoes (Sermwittayawong et al. 2012; Ministry of Public Health of Thailand 2023). The Thai Ministry of Public Health has reported ongoing malaria cases in several border islands, including Chang and Kood in Trat Province near the Thai–Cambodian border, and Phayam and Chang in Ranong Province, close to the Thai–Myanmar border (Ministry of Public Health of Thailand 2023). Furthermore, surveys on these islands have consistently identified *An. baimaii* as the dominant *Anopheles* species involved in the transmission of malaria (Ritthison et al. 2014; Chaiphongpachara et al. 2022b).

The island region's unique ecosystem stemming from relative isolation from the mainland offers insights into evolutionary biology (Chown and Terblanche 2006; Whittaker and Fernández-Palacios 2007). This isolation often leads insects on the island to adapt to their specific environment (Whittaker and Fernández-Palacios 2007), which includes distinctive feeding habits, reproductive strategies, and physical characteristics (Whittaker and Fernández-Palacios 2007; Yi et al. 2016). The genetic and morphological variations observed in insects inhabiting these islands are crucial for understanding the process of evolution, as they exemplify how isolation, when combined with particular environmental pressures, leads to habitat-specific adaptations (Vieira et al. 2016; Petersen et al. 2022). However, research into these variations, especially in mosquito vectors, is still limited in Thailand's island regions. This gap underscores the need for more studies in these distinct environments to better understand the evolutionary dynamics of disease vectors.

Genetic variation plays a pivotal role in the adaptability of insect populations to environmental changes, forming the basis for natural selection (Mopper 1996; Powell 2018). Modern research often focuses on studying genetic variations within natural insect populations (O'Loughlin et al. 2008; Failloux et al. 2002). Geographic isolation can result in reduced gene flow, leading to significant genetic differences (Lanzaro and Tripet 2004; Weeraratne et al. 2018). These variations might escalate the severity of mosquito-borne infectious diseases. Previous studies have linked genetic variation to the susceptibility of mosquitoes to infections and have identified specific DNA variations in mosquito vectors that are associated with insecticide resistance, an important consideration for disease control (Bosio et al. 2000; Verhaeghen et al. 2010; Kabula et al. 2014). The mitochondrial DNA, especially the cytochrome *c* oxidase subunit I gene (*COI*), is frequently used in mosquito population genetic studies due to its higher mutation rate compared to nuclear DNA, making it an effective tool for assessing genetic variation (Allio et al. 2017; Mohapatra et al. 2019).

Morphological adaptations in insects, particularly those in island environments, often involve alterations in wing size and structure, as well as the development of camouflaging features (Sheikh et al. 2017). These phenotypic adaptations are instrumental in facilitating the livelihood and survival of insect species within their distinct ecological niches (Alves et al. 2016; Bai et al. 2016). In entomological research, specifically in mosquitoes, geometric morphometrics (GM) has emerged as a pivotal tool for assessing phenotypic variation based on the morphological differences in wing shape (Wilk-Da-Silva et al. 2018; Chaiphongpachara and Laojun 2020; Martinet et al. 2021; Oliveira-Christe et al. 2023). Wings, crucial for flight, also hold species-specific information that aids in identifying various mosquito species (Lorenz et al. 2017). Recent applications of this technique have successfully differentiated *An. baimaii* from its closely related species, *An. dirus*. This technique underscores the unique wing shapes of *An. baimaii*, distinguishing it from similar species (Chaiphongpachara et al. 2022b). These wing GM analyses offer insights into the morphological traits of mosquito populations. When integrated with genetic data, these analyses significantly enrich our understanding of the population structure of these mosquitoes, providing a more comprehensive view of their biological and ecological characteristics (Hounkanrin et al. 2023).

This study was conducted to better understand the biological variations and *Plasmodium* infection status in *An.*

baimaii, an important malaria vector in Thailand's border islands. A comprehensive approach was employed, consisting of genetic analysis using the mitochondrial *COI* gene and wing phenotypic evaluation through the GM technique. In addition, we conducted screenings for *Plasmodium* parasites in *Anopheles baimaii* samples. The study encompassed four border islands: Chang and Kood islands in Trat Province, along the Thai–Cambodian border, and Phayam and Chang islands in Ranong Province, adjacent to the Thai–Myanmar border. The insights garnered from this research are critical for the development of effective malaria control strategies and in enhancing the understanding of the ecological dynamics of *An. baimaii*, which is vital for addressing future public health challenges in these areas.

Materials and methods

Study area

The research was conducted on four islands located along Thailand's national borders, chosen for their relevance in malaria epidemiology as indicated by the Ministry of Public Health of Thailand (http://malaria.ddc.moph.go.th/malariaR10/index_v2.php). These islands comprise Chang and Kood islands in Trat Province, situated on the Thai–Cambodian border, with geographical coordinates of 12°01′59.8"N, 102°21′48.5"E for Chang island, and 11°39′23.8"N, 102°34′50.9"E for Kood island. Additionally, Chang and Phayam islands in Ranong Province, proximate to the Thai–Myanmar border, are included, positioned at 9°50′03.7"N, 98°27′06.1"E and 9°43′58.0"N, 98°24′23.1"E, respectively (Fig. 1).

Each island presents unique environmental attributes. Chang and Kood islands, integral to the Trat archipelago in eastern Thailand, are distinguished by their ecological tourism potential. These islands are subjected to a mild climate regime, experiencing cool, hot, and rainy seasons with significant precipitation. Chang island, the second-largest island in Thailand and the largest in the Gulf of Thailand, encompasses approximately 212.40 square kilometers (km^2), featuring a predominantly natural rainforest ecosystem and a diverse, mountainous topography. In contrast, Kood island, Thailand's fourth-largest island, is relatively flatter and covers approximately 111.89 km^2.

Conversely, Chang and Phayam islands in the Ranong archipelago, located in southern Thailand, are similarly recognized for their ecological tourism significance. Chang island of Ranong, the largest in this archipelago, spans approximately 20.22 km^2 and is characterized by its mountainous landscape and well-preserved forest regions. Phayam island, the second-largest island in this archipelago, occupies about 16.87 km^2. Its central region is a blend of mountainous terrains and dense forests, with certain areas transformed into agricultural land. The coastal regions of these islands are marked by bays interspersed with rocky formations, gradually transitioning into sandy beaches in the central bay areas. The seasonal dynamics in the Ranong archipelago contrast with those in the Trat archipelago. The islands in Ranong Province experience only two distinct seasons: the hot season, influenced by the southeastern monsoon, from February to May; and the rainy season, driven by the southwest monsoon, with the heaviest rainfall occurring from May to October. Subsequently, the northeastern monsoon lowers temperatures and brings widespread coastal rains from October to February.

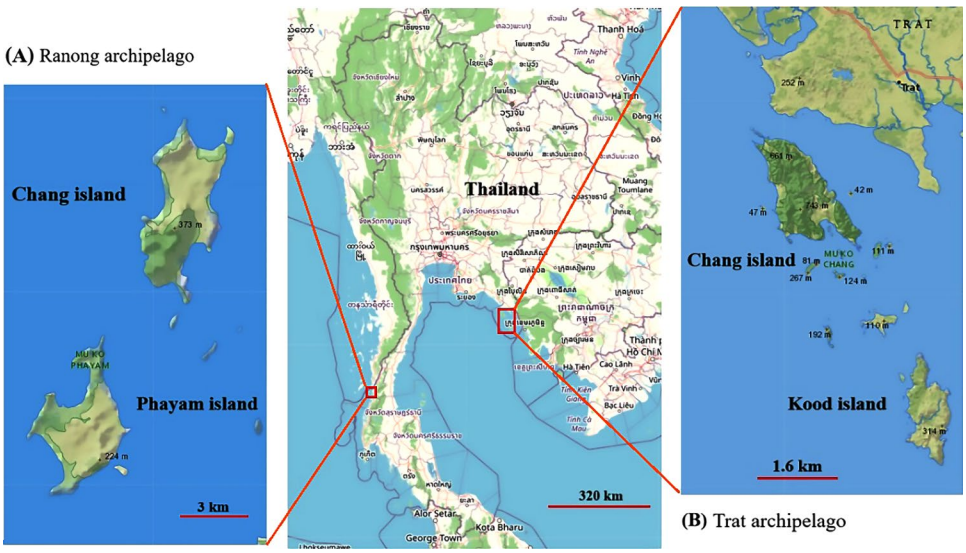

Fig. 1 Map of study sites: (**A**) Chang and Phayam islands, part of the Ranong archipelago in southern Thailand, and (**B**) Chang and Kood islands, part of the Trat archipelago in eastern Thailand. This map was obtained from the USGS National Map Viewer, which is a publicly accessible platform (https://apps.nationalmap.gov/viewer)

Mosquito collection

For the collection of adult mosquitoes, we used the BG-Pro CDC-style mosquito trap (BioGents, Regensbourg, Germany), each outfitted with a Power Bank backup battery, supplemented with dry ice, and equipped with a BG-lure cartridge. A total of 12 traps were deployed per island, strategically positioned within the forested areas and at forest edges. The traps were set approximately 1.5 m above the ground and operated continuously for a period of 10 nights per island during the 2022 rainy season (July–September). This duration was chosen to capture a representative sample of the mosquito population during high abundance.

Mosquito trapping began each evening before sunset, starting at 6:00 p.m., and continued until dawn at 6:00 a.m., resulting in 12 h of operation nightly. In the morning, we carefully removed the insect sampling bags from all traps, and they were then placed in a dry ice storage box at a temperature of -20 °C for 20 min. This procedure was conducted to ensure the euthanasia of any live mosquitoes in the sampling bags. Thereafter, these sample collection bags were transported to the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Campus, where detailed species identification was conducted.

Mosquito identification

For the identification of *An. baimaii*, a sibling species within the *An. dirus* complex, both morphological and molecular methods were used.

Morphological identification

The adult *Anopheles* mosquitoes collected from the field were first identified using standard morphological methods. We referred to the illustrated key to the *Anopheles* mosquitoes of Thailand, as detailed by Rattanarithikul et al. (2006). The identification process involved examining the mosquito samples under an SMZ 800 N stereo microscope (Nikon Corp., Tokyo, Japan). Those mosquito samples that were morphologically classified as the *An. dirus* complex underwent further identification through the multiplex polymerase chain reaction (PCR) method.

Molecular identification

For molecular species identification, we extracted DNA from three to four legs of each mosquito sample identified as belonging to the *An. dirus* complex. This extraction process was conducted using the FavorPrep™ mini kit (Favorgen Biotech, Ping-Tung, Taiwan), strictly adhering to the manufacturer's instructions. Subsequently, we used the extracted DNA from each sample to identify *An. baimaii* using the multiplex allele-specific polymerase chain reaction (AS-PCR) assay. This assay targeted the internal transcribed spacer 2 (ITS2) region, following the methodology established by Walton et al. (1999). Distilled water served as the negative control, while DNA from *An. dirus* s.s. and *An. baimaii*, derived from a prior study (Chaiphongpachara et al. 2022a, b), acted as positive controls. The PCR products of all samples were then subjected to electrophoresis on 2.5% agarose gels to determine the DNA fragment size. These gels were stained with Midori Green (Nippon Gene, Tokyo, Japan). We then examined the fragment sizes using the ImageQuant LAS 500 CCD imager (GE Healthcare Japan Corp., Tokyo, Japan). A DNA fragment size of 306 base pairs (bp) was the key indicator for the identification of *An. baimaii*. Samples that did not display any bands or those with DNA fragment sizes differing from 306 bp were excluded from further analysis in our study.

Plasmodium detection

In the process of identifying the presence of *Plasmodium* species within each *An. baimaii* specimen, DNA extraction was conducted from the head–thorax and abdomen using the FavorPrep™ mini kit. After extraction, genus- and species-specific nested PCR assays targeting the small subunit ribosomal RNA (SSU rRNA) genes were performed to detect *Plasmodium* DNA in accordance with the methodologies delineated by Singh et al. (1999). The primary PCR used *Plasmodium* genus-specific primers: rPLU1 (5′-TCA AAG ATT AAG CCA TGC AAG TGA-3′) and rPLU5 (5′-CCT GTT GTT GCC TTA AAC TCC-3′), following the procedure detailed by Singh et al. (1999). The product from this first PCR (Nest 1) served as the DNA template for the second PCR (Nest 2). In Nest 2, species-specific primers were employed for detecting various *Plasmodium* species: rFAL1/rFAL2 for *P. falciparum*, rVIV1/rVIV2 for *P. vivax*, rOVA1/rOVA2 for *P. ovale*, and rMAL1/rMAL2 for *P. malariae* (Singh et al. 1999). The DNA amplification protocol followed the guidelines of Tassanakajon (Boonsaeng et al. 1993). Both positive and negative controls were included in every PCR, with the positive control comprising DNA from *P. falciparum* and *P. vivax*, extracted from the blood of patients provided by the Vector Borne Disease Control Center, Ranong, Thailand.

Wing geometric morphometrics

The wings of *An. baimaii* samples were prepared as wing slides to examine variations in wing shape among the populations from different border islands using the GM technique. Initially, we selected mosquito samples with intact

wings. The right wing of each mosquito was then carefully detached from the body. After positioning the wing on a slide using a small needle, we mounted it with Hoyer's mounting medium. Any feathers or scales on the wings were gently brushed away to enhance the visibility of the wing pattern. Following this, the slide was covered with a glass coverslip. The prepared slides were then photographed using a digital camera connected to a SMZ 800 N stereo microscope (Nikon Corp., Tokyo, Japan).

Eighteen specific landmarks (Fig. 2.) were digitized on the wing using XYOM version 2 (Dujardin and Dujardin 2019), an online morphometric tool (https://xyom.io). These landmark positions, based on a previous study, have been proven effective for identifying *An. baimaii* (Chaiphongpachara et al. 2022b).

In this study, 20 wings were randomly selected and digitized twice by the same individual to ensure the precision of the digitized landmarks. The precision was assessed using the repeatability (*R*) index (Arnqvist and Mårtensson 1998) through XYOM software. MorphoJ software (Klingenberg 2011) was used for GM analyses. To evaluate the influence of wing size on wing shape variation (allometry), a regression of the Procrustes coordinates (dependent variable) on centroid size (independent variable) was conducted. This analysis included a permutation test with 10,000 permutations, setting the threshold for statistical significance at $p < 0.05$. After conducting the regression, we used the residuals from the regression of shape on size to test for differences in shape, removing the influence of size (allometry-free variables). This approach allowed us to analyze wing shape variations in *An. baimaii* independently of wing size.

Canonical variate analysis (CVA) was used to explore the variation in wing shape across the study populations. Additionally, this analysis involved calculating the Mahalanobis distances to estimate the metric distances between different groups. To ascertain the statistical significance of wing shape differences among the populations, we conducted permutation tests with 10,000 permutations based on the Mahalanobis distances. The threshold for statistical significance was established at $p < 0.05$. For a detailed examination of the wing shape variation patterns among the island populations, we constructed a single-linkage clustering tree. This tree, based on the Mahalanobis distances, was built using PAST V.4.01 software (Hammer et al. 2001).

Molecular analyses

The DNA previously extracted from the legs of *An. baimaii* for species identification was reused to examine genetic variation based on the mitochondrial *COI* gene. The same samples employed in GM analyses were used for this genetic investigation. The samples used for genetic investigating are the same samples used for GM analyses. For the amplification of 709 bp of the *COI* gene, we used the primers *COI*_F (forward primer: 5′-GGA TTT GGA AAT TGA TTA GTT CCT T-3′) and *COI*_R (reverse primer: 5′-AAA AAT TTT AAT TCC AGT TGG AAC AGC-3′) (Kumar et al. 2007). The PCR reaction was prepared in a total volume of 25 µL, which included 4 µL of DNA template, 0.2 µM of each primer, 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 5% dimethyl sulfoxide. We also incorporated 1.5 U of Platinum Taq DNA polymerase (Invitrogen) into the mixture, and the final volume was achieved with the addition of distilled water. The PCR conditions were conducted in accordance with the procedures established in a previous study by Chaiphongpachara et al. (2022a). Following amplification, the PCR products from all samples underwent electrophoresis on 1% agarose gels to verify the quality of the PCR products. All PCR products that met the quality standards were sent to SolGent, Inc. (Daejeon, Korea) for further purification and sequencing.

The *COI* gene sequences from *An. baimaii* specimens were carefully analyzed. We first manually

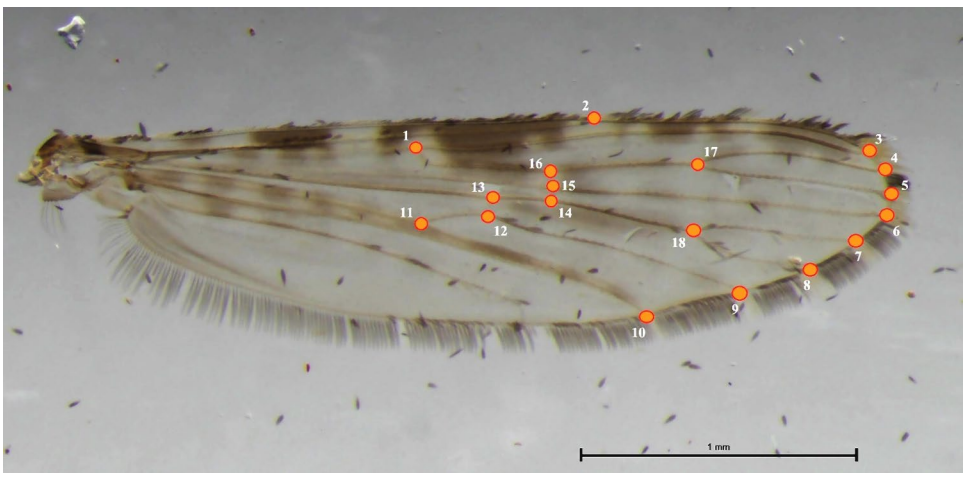

Fig. 2 Right wing of *An. baimaii*, illustrating the 18 landmarks used for geometric morphometric (GM) analyses in this study

edited the trace files (chromatograms) and then generated a consensus sequence for each specimen using BioEdit software (Hall 1999). Following this, we estimated various genetic diversity indices, including the number of segregating sites (s), nucleotide diversity (π), the number of haplotypes (h), haplotype diversity (Hd), and average nucleotide differences (k), using DnaSP software version 6 (Rozas et al. 2017). Neutrality tests were conducted to explore potential recent population expansions. These included Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989), which were also performed using DnaSP software version 6. A mismatch analysis was performed using DnaSP to compare the observed frequency distribution of pairwise differences among haplotypes against the expected distribution in a model of population expansion. The smoothness of the mismatch distribution was assessed by calculating the raggedness (r) statistic, and the significance of this statistic was evaluated using 10,000 bootstrap replicates.

For evaluating population structure within and among populations of *An. baimaii*, the analysis of molecular variance (AMOVA) was performed with 10,000 permutations in Arlequin software version 3.5.2.2 (Excoffier 2010). We calculated the pairwise fixation index (F_{st}) between populations using haplotype frequencies to estimate genetic differentiation, using Arlequin software. The significance of these measures was tested with 10,000 permutations. A single-linkage clustering tree was constructed to investigate the patterns of closest genetic relationships among *An. baimaii* populations from four border islands. This tree, based on F_{st} values, was generated using the PAST software. To illustrate the genetic diversity and geographic relationships among the *An. baimaii* populations observed, we created a haplotype network. This network was generated using the median-joining network method in PopArt software version 1.7 (Leigh and Bryant 2015). All *An. baimaii* sequences obtained in this study have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers OR794397 to OR794468.

Results

During this study, a total of 119 *An. dirus* complex specimens were collected from two archipelagos, encompassing four islands. The multiplex AS-PCR assay identified 19 individuals as *An. dirus* s.s. and 100 individuals as *An. baimaii*. All 19 *An. dirus* s.s. individuals were collected from Chang island within the Trat archipelago. Table 1 further details the distribution and number of *An. baimaii* specimens used for both wing physiology and genetic variation analyses. Additionally, our tests using nested PCR assays did not detect any of the four *Plasmodium* malaria parasites (*P. falciparum, P. malariae, P. vivax*, and *P. ovale*) in all the *An. baimaii* samples.

Wing shape variation

Before conducting the GM analyses, we confirmed the high precision of digitizing the landmarks within our image set, achieving a 97% accuracy rate for shape, as indicated by the repeatability test. A regression analysis of the Procrustes coordinates on the centroid sizes of *An. baimaii* samples revealed that the allometric effect accounted for 5.57% of the total shape variation. This was statistically significant ($p < 0.05$) based on 10,000 permutations, as analyzed in MorphoJ software.

The results of wing shape analysis were visualized by CVA, of which allometry-free variables revealed the variation between population groups (Fig. 3). The CVA revealed three canonical variates (CV), with the first two explaining 89.78% of the total variation (CV1: 65.56% and CV2: 24.22%). When we statistically evaluated the wing shape differences based on Mahalanobis distances, significant variations were observed in nearly all populations ($p < 0.05$). The only exception was observed between the *An. baimaii* populations of Chang and Phayam islands in Ranong Province, which did not show a significant difference ($p > 0.05$), as presented in Table 2.

The single-linkage clustering tree, constructed using Mahalanobis distances, effectively demonstrates the wing

Table 1 Total number of *An. baimaii* collected in the islands and the number of specimens used for both geometric morphometric (GM) and genetic analyses

Location	Total number collected in the islands	Number used for GM analysis	Number used for genetic analysis
Chang island (Trat)	26	25	19
Kood island (Trat)	40	39	25
Chang island (Ranong)	18	17	16
Phayam island (Ranong)	16	16	12
Total	100	97	72

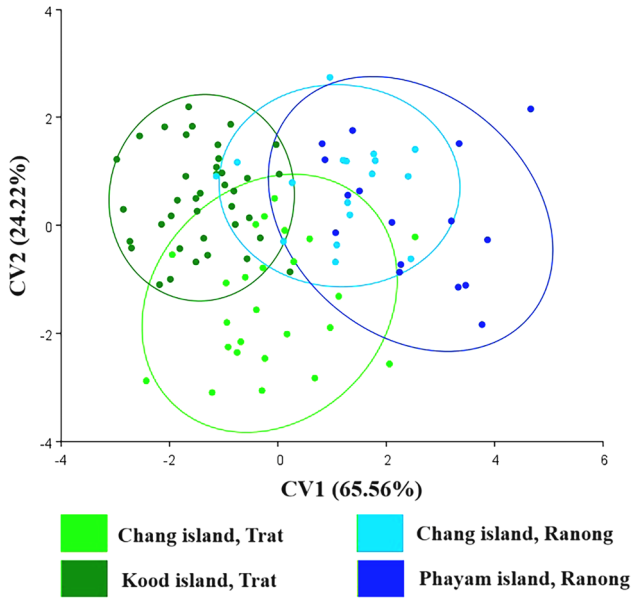

Fig. 3 Canonical variate analysis (CVA) of *An. baimaii* populations from four different border islands in Thailand

Table 2 Pairwise comparisons of *An. baimaii* populations from four Thai border islands, using Mahalanobis distances as determined by geometric morphometric (GM) analyses

Population	Chang island (Trat)	Kood island (Trat)	Chang island (Ranong)	Phayam island (Ranong)
Chang island (Trat)	0			
Kood island (Trat)	2.35*	0		
Chang island (Ranong)	2.68*	2.84*	0	
Phayam island (Ranong)	3.23*	3.91*	2.30	0

An asterisk (*) after a pairwise Mahalanobis distance value indicates that the pairs are statistically different at $p < 0.05$

shape similarities among *An. baimaii* populations from four Thai border islands, as shown in Fig. 4. This tree categorizes the populations into two primary clusters according to their geographical locations. The first cluster comprises populations from the islands in Trat Province, which borders Cambodia. Meanwhile, the second cluster includes populations from the islands in Ranong Province, located near the Myanmar border.

Genetic variation

In this study, the genetic variation of *An. baimaii* was assessed by analyzing *COI* gene sequences from 72 samples. The analysis of these 72 *COI* gene sequences from

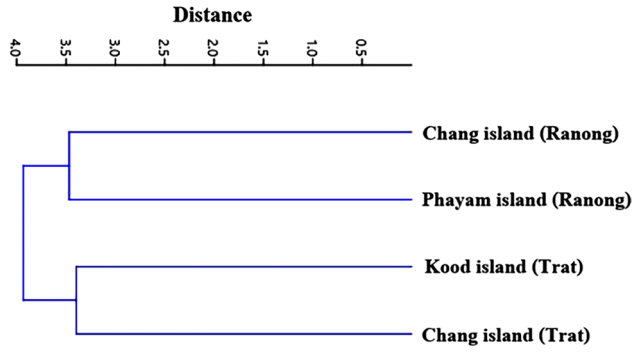

Fig. 4 Single-linkage clustering tree illustrating wing shape similarities of *An. baimaii* populations from four border islands in Thailand

four *An. baimaii* populations identified 22 segregating sites and revealed a relatively low nucleotide diversity (π) of 0.004 ± 0.001. This indicates limited genetic variation within the *COI* gene among these populations. Additionally, 18 distinct haplotypes were identified, demonstrating a high haplotype diversity (*Hd*) of 0.825 ± 0.037.

The genetic diversity indices for each *An. baimaii* population are detailed in Table 3. All populations showed high haplotype diversity compared to the nucleotide diversity. This pattern commonly suggests recent expansions of these populations from a relatively small number of ancestors. Neutrality tests conducted on the overall population yielded negative values: Fu's *Fs* was -5.029, and Tajima's *D* was -1.028. Such negative values are typically indicative of population expansion or selective sweeps. However, Tajima's *D* did not reach statistical significance ($p > 0.05$). The mismatch distribution analysis revealed a bimodal pattern, as illustrated in Fig. 5. Furthermore, with a raggedness index of 0.068, no significant difference was observed between the observed and simulated data ($p = 0.485$).

An AMOVA was performed on *An. baimaii* populations to understand their genetic structure. The analysis, detailed in Table 4, revealed that 82.68% of the genetic variation occurred within populations, while 17.32% occurred among populations. The F_{st} value, reported at 0.173, was statistically significant ($p < 0.05$), suggesting notable genetic differentiation among the populations. Further assessments of pairwise genetic differentiation revealed significant differences between all populations ($p < 0.05$), as detailed in Table 5.

Figure 6 displays a haplotype network for *An. baimaii*, illustrating the genetic relationships and diversity across four distinct population groups. This network encompasses 18 unique haplotypes. Haplotype H1 standing out as the most prevalent, found in 27 samples from all four islands. The majority of the haplotypes were specific to their locations, comprising 13 haplotypes (H5–H12, H14–H18). Meanwhile, five

Table 3 Genetic diversity indices and neutrality tests (Fu's *Fs* and Tajima's *D*) based on the *COI* sequences of *An. baimaii* populations from four border islands in Thailand

Population		*n*	*s*	π (±SD)	*h*	Hd (±SD)	*k*	Neutrality tests	
								Fu's *Fs*	Tajima's *D*
Trat	Chang island	19	8	0.002 ± 0.001	4	0.456 ± 0.132	1.427	0.785	-1.288
	Kood island	25	16	0.005 ± 0.001	11	0.847 ± 0.058	3.293	-2.503*	-0.786
Ranong	Chang island	16	8	0.003 ± 0.001	5	0.667 ± 0.113	2.475	0.879	0.095
	Phayam island	12	11	0.005 ± 0.001	7	0.909 ± 0.003	3.985	-0.604	0.012
Overall		72	22	0.004 ± 0.001	18	0.825 ± 0.037	3.160	-5.029*	-1.028

An asterisk (*) after the neutrality test value indicates statistical significance. Abbreviations: *n* = number of sequences; *s* = number of segregating sites; *π* = nucleotide diversity; *h* = number of haplotypes; *Hd* = haplotype diversity; *k* = average number of nucleotide differences; SD = standard deviation

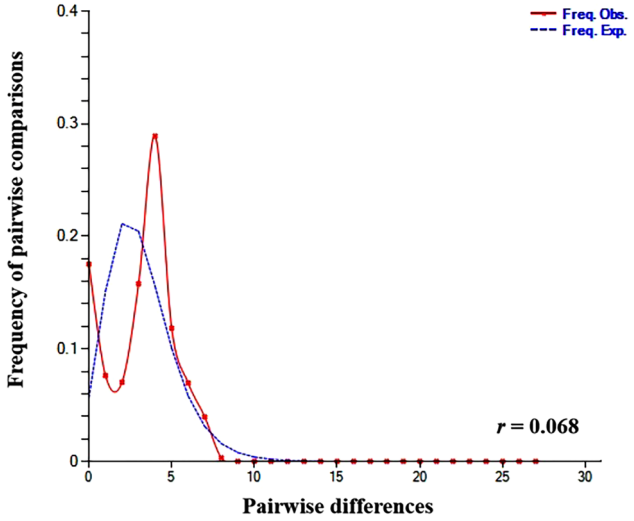

Fig. 5 Mismatch distribution graph for *An. baimaii* populations from Thailand's border islands. The X and Y-axis display the number of pairwise differences and the frequency of the pairwise comparisons, respectively. Observed frequencies are illustrated with a solid red line, while the expected frequency, based on the hypothesis of a constant population model, is shown with a blue dotted line

haplotypes (H1–H4, H13) are shared across different locations. The single-linkage clustering tree (Fig. 7), which depicts the genetic similarities among the *An. baimaii* populations from four Thai border islands based on the F_{st} values, suggests that populations from Kood island (Trat) and Phayam island (Ranong) are genetically closer to each other than to those from the other islands.

Discussion

Anopheles baimaii is recognized as an important malaria vector for *P. falciparum* and *P. vivax* (Rattanarithikul et al. 1996; Prakash et al. 2005; Sinka et al. 2011). In Thailand, this *Anopheles* species is largely limited to the central, southern, and western regions, especially along the Thai–Myanmar border (Tainchum et al. 2015). Notably, its presence has also been documented on the Thai–Cambodian border islands, including Chang (Ritthison et al. 2014) and Kood (Chaiphongpachara et al. 2022b) islands. Our study extends the known range of *An. baimaii* to include not only the Chang and Kood islands of Trat Province, Thai–Cambodian border, but also the Chang and Phayam islands of Ranong Province, Thai–Myanmar border. Although no *Plasmodium* infections were detected in our mosquito samples, the presence of this critical malaria vector in border regions emphasizes the imperative for ongoing surveillance by healthcare authorities. This proactive measure is essential given the risk of malaria being introduced into the area by migrant workers (Kitvatanachai and Rhongbutsri 2012) and the possibility that tourists could spread the disease to other regions (Froeschl et al. 2018).

In this study, the landmark-based GM technique was used to investigate variations in the wing shape of *An. baimaii*. We acknowledge that wing shape is genetically associated with the mosquito species (Dujardin 2011; Lorenz et al. 2017). Consequently, the GM analysis specifically focused on shape rather than size. This focus was due to their high sensitivity to environmental conditions at larval breeding sites (Dujardin 2011; Lorenz et al. 2017; Phanitchat et al.

Table 4 Analysis of molecular variance (AMOVA) of *An. baimaii* populations from four border islands in Thailand

Source of variation	d.f	Sum of squares	Variance components	% of variation	F_{st}	*p*-value
Among populations	3	5.013	0.075	17.32	0.173	< 0.05
Within populations	68	24.265	0.357	82.68		
Total	71	29.278	0.432	100		

Table 5 Pairwise genetic differentiation (F_{st}) among *An. baimaii* populations from four border islands in Thailand

Population		Pairwise F_{st}			
		Trat Province		Ranong Province	
		Chang island	Kood island	Chang island	Phayam island
Trat	Chang island	-			
	Kood island	0.096*	-		
Ranong	Chang island	0.293*	0.158*	-	
	Phayam island	0.284*	0.097*	0.196*	-

An asterisk (*) signifies significant pairwise genetic differentiation. F_{st} values close to 0 suggest minimal differentiation between populations, whereas values closer to 1 indicate more substantial genetic differentiation

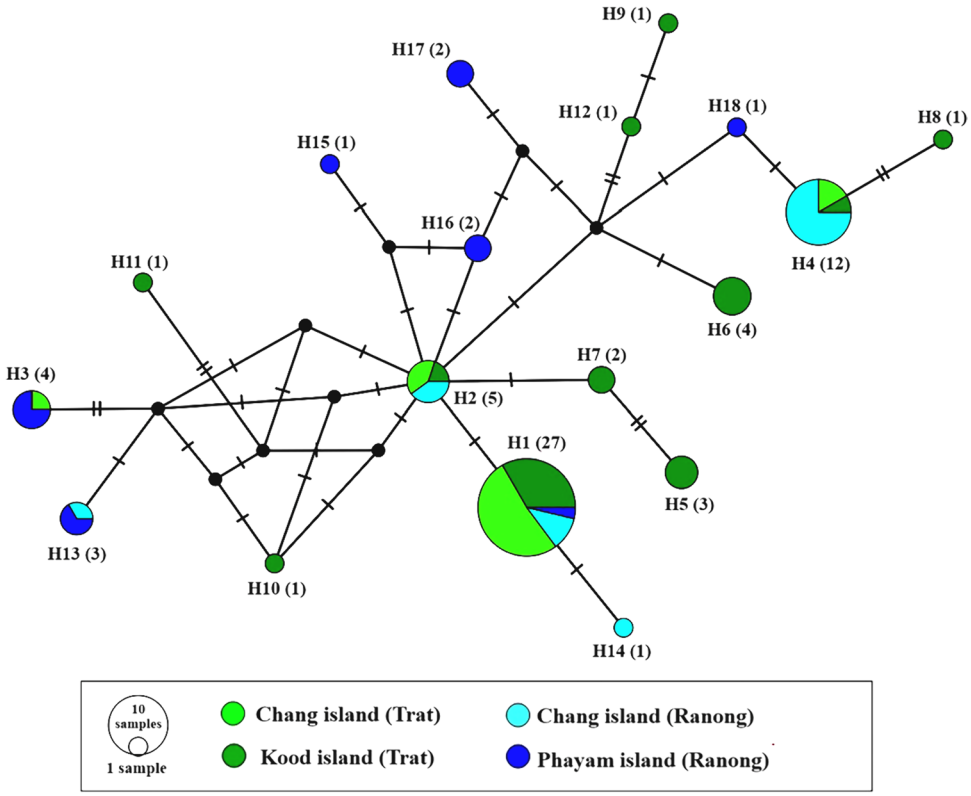

Fig. 6 Haplotype network of *An. baimaii* populations from Thailand's border islands. In this visualization, haplotypes from each location are represented by circles in distinct colors. The size of each circle is proportional to the total number of *COI* sequences for that particular haplotype. Additionally, the number of mutations is indicated by dash marks along the connecting lines between haplotypes

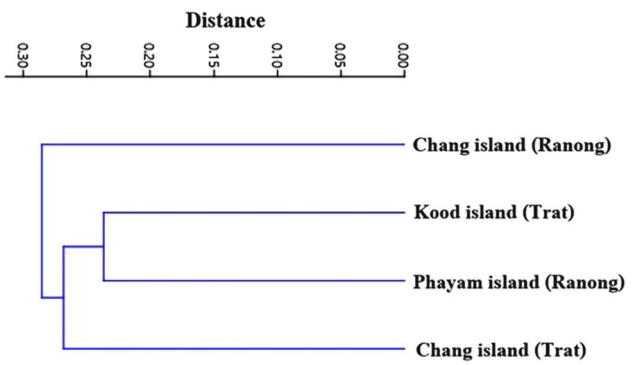

Fig. 7 Single-linkage clustering tree illustrating genetic similarities among *An. baimaii* populations from four border islands in Thailand, based on the fixation index (F_{st})

2019), which complicates their assessment across broader scales such as geographical variation (Morales Vargas et al. 2021). While it has been previously suggested that temperature might influence size, the variability induced by seasonal temperature changes renders this size variation unstable (Hidalgo et al. 2015; Barr et al. 2023). Supporting this, a recent study on geographic populations of *Culex tritaeniorhynchus* revealed no significant correlation between wing size and geographic variation. This is in contrast to wing shape, which shows a clear connection to geography, driven by genetic factors (Morales Vargas et al. 2021).

Further allometric analysis showed a significant influence of wing size on shape. To accurately assess within-species variation and avoid deviations caused by allometric effects,

it is crucial to eliminate these effects on shape (Klingenberg 1996; Dujardin 2011). Therefore, in our analyses, we used allometry-free variables, ensuring that the shape variable was independent of size effects. The examination of wing shape variation across four island populations revealed significant differences in most comparisons. However, Chang and Phayam islands in Ranong Province showed no statistical difference in wing shape ($p > 0.05$), likely owing to their geographical proximity. This is corroborated by a single-linkage clustering tree derived from Mahalanobis distances, illustrating that islands closer together tend to have more similar wing shapes while more distant islands form a distinct group. The two archipelagos studied are approximately 500 km apart and are situated in different regions of Thailand. The islands in Ranong Province are located in the Andaman Sea, part of the Indian Ocean, while the islands in Trat Province are in the Gulf of Thailand, connected to the Pacific Ocean and the South China Sea (Koad et al. 2012). These geographical distinctions result in different climates and ecosystems, which are reflected in the wing shape variations observed in the *An. baimaii* population, supported by a single-linkage clustering tree.

The Chang and Kood islands in Trat, characterized by their diverse rainforest plant range away from the coast, contrast with the smaller Chang and Phayam islands in Ranong, which, despite having fewer plant varieties, still predominantly feature forested areas. Furthermore, the weather conditions in these provinces differ significantly. Trat experiences an average annual temperature of 27.36 °C, a total annual rainfall of 3,073.4 mm, and an average wind speed of 5.30 mph. In contrast, Ranong has a cooler average annual temperature of 26.99 °C, less annual rainfall at 2,405.38 mm, and a lower average wind speed of 4.33 mph (Weatherspark 2023). Previous research has shown that different climates and ecosystems can lead to variations in wing shape among various mosquito species, including *Aedes aegypti* (Wilk-Da-Silva et al. 2018), *Anopheles coluzzii* (Hidalgo et al. 2015), *Anopheles darlingi* (Motoki et al. 2012), *Culex nigripalpus* (De Carvalho et al. 2017), and *Culex tritaeniorhynchus* (Morales Vargas et al. 2021).

The genetic diversity indices in this study provided insights into the genetic profiles of *An. baimaii* populations. Across all populations, the haplotype diversity, ranging from 0.456 to 0.909, exceeded the nucleotide diversity, which was between 0.002 and 0.005. This suggests that these populations have recently expanded from a smaller, genetically similar group. Such a pattern is common in isolated populations, like those on islands, where initial genetic variation might have been limited due to founder effects or population bottlenecks. As the population grows over time, new haplotypes emerge, thereby increasing haplotype diversity. However, these new haplotypes have not yet had enough time to accumulate significant nucleotide level differences, resulting in a low overall nucleotide diversity (Jamieson 2011; Li et al. 2020).

Neutrality tests, including Fu's *Fs* and Tajima's *D*, were used to assess if the genetic diversity within *An. baimaii* populations diverge from what is typically expected under neutral evolution. Negative values in both Fu's *Fs* and Tajima's *D* suggest potential recent population expansions. However, only Fu's *Fs* achieved statistical significance. This is because Fu's *Fs* is particularly sensitive to recent population expansions, especially those involving numerous low-frequency alleles (Ramos-Onsins and Rozas 2002; Zhang et al. 2022). On the other hand, Tajima's *D*, being more conservative, may not effectively detect certain demographic shifts or selective pressures (Ramos-Onsins and Rozas 2002). The lack of statistical significance in Tajima's *D* indicates that, while a recent expansion is suggested by Fu's *Fs*, it may not have been substantial enough to significantly alter the balance between the number of segregating sites and the average nucleotide differences. This aligns with a previous study examining the genetic diversity of this mosquito in mainland Thailand, which reported a similar level of genetic diversity to that found in the island areas of our study. This suggests that the populations do not experience severe genetic bottlenecks (O'Loughlin et al. 2008). However, this study observed a bimodal mismatch distribution pattern. This outcome aligns with previous research that identified similar patterns in *Aedes aegypti* in Penang, Malaysia (Md Naim et al. 2020), and in *Anopheles minimus* across China, Thailand, and Vietnam (Chen et al. 2011). These studies proposed that the bimodal mismatch distribution observed in the populations is likely due to the low migration rate of this malaria vector between the studied islands. To substantiate this hypothesis, future research should employ additional gene markers and larger sample sizes for validation. Furthermore, the studied populations exhibited a non-significant raggedness index ($p > 0.05$), suggesting that the data fits well with a model of population expansion.

An AMOVA-based analysis of the population structure of *An. baimaii* in Thailand's border islands revealed that 17.32% of the genetic variation existed among populations. This was further supported by significant genetic differentiation observed among populations when pairwise genetic differences were evaluated using F_{st} values ($p < 0.05$). Several factors contribute to this variation. First, limited dispersal and isolation have a notable impact on *An. baimaii* populations across different islands, leading to genetic differentiation (Frankham 1997). Second, the founder effect and genetic drift are particularly influential in island populations. A small number of individuals colonizing a new area can lead to a population with initially limited genetic variation. Over time, genetic drift, characterized by random changes in allele frequencies, can introduce significant variability within these populations (Barton 1996; Frankham 1997).

Last, the diverse environmental conditions unique to each island exert various selective pressures. These pressures may lead to the development of distinct genetic traits within each population, thereby enhancing genetic diversity as populations adapt to their specific environments (Chen et al. 2004; Whittaker and Fernández-Palacios 2007). Our findings align with those from a previous study examining the population structure of *Anopheles gambiae* on four islands in Uganda. That study reported significant genetic differences among the island populations, attributing these variations to factors such as geographical separation by water, the dynamics of small populations, and local adaptation processes (Kayondo et al. 2005). Furthermore, molecular investigations into *Anopheles hinesorum* and *Anopheles farauti* in the Solomon archipelago have shown that differences in host preferences among geographically separated populations may influence the genetics of mosquitoes on each island (Ambrose et al. 2012, 2021). Human colonization of the islands also plays a role in genetic variation (Ambrose et al. 2021).

The haplotype network for *An. baimaii* displayed the genetic relationships among haplotypes from four distinct locations. Haplotype H1, the most prevalent, is found in 27 samples and is shared across all locations, suggesting its potential as an ancestral or widespread haplotype. The predominance of location-specific haplotypes indicates genetic isolation or restricted gene flow among the islands. The absence of clear geographical clustering in the network hints at possible gene flow or a recent shared ancestry among the populations. Shared haplotypes, such as H1, H2, and H4, which are present in multiple locations, suggest historical or ongoing genetic exchange. However, the existence of unique haplotypes at each site also points to genetic differentiation, likely a result of the islands' geographic isolation. Such isolation restricts gene flow and contributes to the distinctive genetic characteristics observed in these island populations (Frankham 1997).

When examining the results for wing shape and genetic variation in *An. baimaii* populations, consistent differences were observed across almost all island populations (with significant pairwise differences, $p < 0.05$). However, the patterns of similarity varied (Fig. 4 vs. Fig. 7). Wing shape diverged according to geographic location, suggesting that environmental factors and adaptive requirements specific to each locale influenced these morphological traits (Morales Vargas et al. 2021). In contrast, genetic variations might reflect historical events such as population bottlenecks or founder effects (O'Loughlin et al. 2008), which might not directly correspond with current geographic distances. Additionally, morphological traits such as wing shape can evolve rapidly in response to environmental pressures (Hidalgo et al. 2015; Barr et al. 2023), whereas genetic differences often accumulate more gradually over time. The genetic markers used in this study likely capture both recent and historical relationships (O'Loughlin et al. 2008). This disparity between morphological and genetic data highlights the complex interplay of evolutionary processes influencing these mosquito populations.

This study has some limitations. Although the *COI* gene has a higher mutation rate compared to nuclear DNA, there are some instances where mitochondrial and nuclear genes display different patterns or relationships. Consequently, future research should explore additional genes to fill in these gaps and provide a more comprehensive understanding.

Conclusion

This study on *An. baimaii* uncovered significant morphological and genetic differences within and between populations across various island locales. The observed variations in nearly all island populations suggest that geographical isolation is key in promoting differentiation. The finding showed the greater haplotype diversity compared to nucleotide diversity, suggesting recent population expansions from a limited genetic base. Neutrality tests yielded a significantly negative Fu's *Fs* value, suggesting a population expansion, while Tajima's *D* did not reach statistical significance. The populations demonstrated a non-significant raggedness index, consistent with a model of population expansion. Additionally, wing shape variations across all islands, grouped according to geography, imply a direct influence of environmental conditions. These findings are essential for understanding the population dynamics of *An. baimaii*. Despite the absence of *Plasmodium* infection in the samples studied, the presence of this significant malaria vector underscores the need for continuous monitoring. The evidence presented underscores the potential for physiological changes in mosquito vectors that could substantially affect malaria transmission. Therefore, it is crucial for health authorities to integrate these insights into the formulation and implementation of vector management and malaria prevention strategies.

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Authors contribution Sedthapong Laojun: conceptualization, methodology, formal analysis, investigation, writing original draft, writing review and editing, visualization; Tanasak Changbunjong: conceptualization, methodology, formal analysis, writing – original draft, writing review and editing, visualization; Tanawat Chaiphongpachara: conceptualization, methodology, formal analysis, investigation, writing original draft, writing review and editing, visualization, supervision.

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Data availability Seventy-two mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene sequences of *Anopheles baimaii* have been uploaded to the GenBank Database (https://www.ncbi.nlm.nih.gov/nuccore) and are available under the accession numbers OR794397 to OR794468.

Declarations

Ethical approval The research protocol, encompassing mosquito collection and animal experimental procedures, was subjected to review and approved by the Animal Ethical Committee of Suan Sunandha Rajabhat University in Bangkok, Thailand. This approval was granted under the application number IACUC 65–003/2022.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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