Infection, Genetics and Evolution 112 (2023) 105452

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular and morphometric differentiation of secondary filariasis vector *Coquillettidia* mosquitoes (Diptera: Culicidae) in Thailand

Sedthapong Laojun [a], Tanasak Changbunjong [b,c], Suchada Sumruayphol [d], Tanawat Chaiphongpachara [a,*]

[a] Department of Public Health and Health Promotion, College of Allied Health Sciences, Suan Sunandha Ra-jabhat University, Samut Songkhram 75000, Thailand
[b] Department of Pre-Clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand
[c] The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals (MoZWE), Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand
[d] Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

ARTICLE INFO

Keywords:
Molecular analysis
Geometric morphometrics
Coquillettidia crassipes
Coquillettidia nigrosignata
Coquillettidia ochracea

ABSTRACT

Coquillettidia mosquitoes are important nuisance-biting pests and a vector of brugian filariasis in Thailand. However, comprehensive information about these mosquitoes remains unavailable such as molecular and morphometric differences among species. The lack of vector knowledge on *Coquillettidia* species could affect future disease control. This study aims to investigate differences in molecular variations based on mitochondrial cytochrome oxidase subunit I (*COI*) gene and wing geometric traits of three *Coquillettidia* species, namely *Cq. crassipes, Cq. nigrosignata,* and *Cq. ochracea* in Thailand. The results of molecular analyses revealed the differences among three *Coquillettidia* species. The genetic difference measure based on the Kimura two-parameter model among three *Coquillettidia* species showed low intraspecific distances (0%–3.05%) and large interspecific distances (10.10%–12.41%). The values of intra- and inter-genetic differences of three *Coquillettidia* species did not overlap which showed the existence of a barcoding gap indicating the efficiency of the identification based on the *COI* gene. As with molecular analysis, the landmark-based geometric morphometrics approach based on wing shape analysis indicated three distinct species groups which were supported by the high total performance score of cross-validated classification (97.16%). These results provide the first evidence of taxonomic signal based on molecular and wing geometric differences to support species identification and biological variations of *Coquillettidia* mosquitoes in Thailand for understanding these rare vector mosquitoes in depth and leading to effective further mosquito control.

1. Introduction

Coquillettidia Dyar, 1905 (Diptera: Culicidae) currently include 58 formally recognized species (Harbach, 2023). The genus *Coquillettidia* is divided into three subgenera, including *Austromansonia* (1 species), *Rhynchotaenia* (44 species), and *Coquillettidia* (13 species) (Harbach, 2023). In adult stage, these *Coquillettidia* species are yellowish medium mosquitoes with narrow symmetrical wing scales and unicolourous (Rattanarithikul et al., 2006; Nugroho et al., 2020). In the immature stages (the larval and pupal stages), all *Coquillettidia* species obtain oxygen for respiration by piercing the roots of aquatic plants using the spiracular apparatus and siphon, uniquely developed for larvae, and pointed trumpets for pupae, which are similar to those of *Mansonia* and *Mimomyia* mosquitoes (Sérandour et al., 2011). Thus, the immature forms of *Coquillettidia* rarely appear on the water surface, but are often attached to the root tissues of various plants in marshes, swamps, and ponds. Most member species in the genus *Coquillettidia* are widely found in the Afrotropical region, including Sub-Saharan Africa, the Nile Basin, and Madagascar (Harbach, 2023). However, some *Coquillettidia* species can be found in different regions, such as the Neotropical, Oriental, Palaearctic and Australasian Regions, and North America (Harbach, 2023). Despite their importance, the bionomics of most species in the genus *Coquillettidia* remain poorly understood.

Several studies have reported that *Coquillettidia* mosquitoes are

* Corresponding author at: Department of Public Health and Health Promotion, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand.
E-mail address: tanawat.ch@ssru.ac.th (T. Chaiphongpachara).

https://doi.org/10.1016/j.meegid.2023.105452
Received 18 March 2023; Received in revised form 13 May 2023; Accepted 22 May 2023
Available online 29 May 2023

important nuisance-biting pests, and some species have been shown to be vectors of many human and animal pathogens. *Coquillettidia perturbans* (Walker, 1856) is a potential vector of Eastern equine encephalitis in horses and humans (Moncayo et al., 2000; Shepard et al., 2016; Sherwood et al., 2020). *Coquillettidia crassipes* (van der Wulp, 1881) is a secondary vector of brugian filariasis in humans, which is caused by *Brugia malayi* (Brug, 1927) (Chiang et al., 1986). Three *Coquillettidia* species, *Cq. aurites* (Theobald, 1901), *Cq. metallica* (Theobald, 1901), and *Cq. pseudoconopas* (Theobald, 1910) are natural vectors of avian malaria parasites in Africa (Njabo et al., 2009).

Thailand is a Southeast Asian country with a tropical rainforest climate and one of the most epidemic prone areas of mosquito-borne diseases, such as Zika virus, chikungunya, dengue, filariasis, Japanese encephalitis, and malaria, due to the high diversity of mosquito vector species (Thongsripong et al., 2013). Four *Coquillettidia* species, including *Cq. crassipes, Cq. nigrosignata* (Edwards, 1917), *Cq. novochracea* (Barraud, 1927), and *Cq. ochracea* (Theobald, 1903) were previously reported in Thailand (Rattanarithikul et al., 2006). *Coquillettidia crassipes* is widely distributed in Thailand. Whlie *Cq. nigrosignata, Cq. novochracea*, and *Cq. ochracea* are rare mosquitoes and their distribution is hardly reported (Rattanarithikul et al., 2006). However, two rare species, *Cq. nigrosignata* and *Cq. ochracea*, as well as *Cq. crassipes* were reported in Narathiwat Province, southern Thailand (Apiwathnasorn et al., 2009). In addition, *Cq. crassipes* can also be found in other countries including Pakistan, India, Sri Lanka, China, Myanmar, Thailand, Lao, Cambodia, Malaysia, Philippines, Papua New Guinea, and Indonesia (Sousa et al., 2000; Vythilingam et al., 2006; Nugroho et al., 2020; Maquart et al., 2021). *Coquillettidia nigrosignata* and *Cq. ochracea* are also found in China, Japan, Cambodia, Philippines, Malaysia, Singapore, and Indonesia (Nugroho et al., 2020; Maquart et al., 2021). Previously, Iyengar (1953) surveyed mosquitoes for *B. malayi* infection in southern Thailand and found one infected (positive) *Cq. crassipes* sample. However, comprehensive information about these mosquitoes remains unavailable such as molecular and morphometric differences among species. The lack of vector knowledge on *Coquillettidia* species can negatively affect future disease control measures.

Genetic and morphological knowledge of mosquito vectors are important for understanding the unique identity of each species (Chan et al., 2014). The mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene fragment, also known as the barcoding sequence, is a widely applied standard DNA marker for species identification in animals including mosquitoes (Cywinska et al., 2006; Wang et al., 2012; Talaga et al., 2017). In addition, *COI* gene has been used as an important genetic marker for determining the intraspecific genetic relationship in numerous mosquito species (Feng et al., 2017; Joyce et al., 2018; Bourke et al., 2021). Similarly, the geometry of the mosquito wing based on geometric morphometric (GM) analysis is gaining popularity for supporting species identification and investigation of morphological variations caused by environmental influences in numerous medically important insects (Lorenz et al., 2017; Chaiphongpachara and Laojun, 2020; Saiwichai et al., 2023). Taxonomic signals have recently been detected in numerous mosquitoes in Thailand using this technique, including in some *Aedes* (Chonephetsarath et al., 2021) and *Anopheles* species (Chatpiyaphat et al., 2021; Chaiphongpachara et al., 2022a).

This study aims to investigate the molecular differences based on *COI* gene and wing geometric traits of three *Coquillettidia* species, including *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* from Narathiwat Province (the area where all three *Coquillettidia* mosquitoes have been reported in Thailand [Apiwathnasorn et al., 2009]). The study assessed the taxonomic signal of *Coquillettidia* in Thailand to understand these rare vector mosquitoes in depth for future effective mosquito control.

2. Materials and methods

2.1. Ethical statement

This study was performed following the guideline of animal care at the Suan Sunandha Rajabhat University, Thailand. The ethical approval for mosquito collections in the field and all scientific procedures in the present study were obtained from the ethics committee of Suan Sunandha Rajabhat University, Bangkok, Thailand (Approval No. IACUC 64–007/2021).

2.2. Mosquito collection and morphological identification

Adult *Coquillettidia* mosquitoes were collected from Narathiwat Province, Thailand (Figs. 1, 6°21'15.7"N, 101°53'36.9"E), following a previous mosquito survey report (Apiwathnasorn et al., 2009). The mosquito collection was carried out during the rainy season when their occurrence is most abundant from August to October 2021, between 6.00 p.m. and 6.00 a.m. for seven consecutive nights per month. A total of 10 BG-Pro CDC-style traps (BioGents, Regensbourg, Germany) with solid carbon dioxide were used and placed 1.5 m above the ground around houses and cattle pens. The next morning, sample bags were removed from the traps and stored in the freezer at −20 °C. Then, at the end of the seven nights of each collection month, all samples were carefully transported to the biological laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Thailand for subsequent experimental steps.

Species identification of female *Coquillettidia* mosquitoes was performed using the classical taxonomic approach based on morphological characters under a Nikon SMZ 800 N stereo-microscope (Nikon Corp., Tokyo, Japan), following the illustrated keys to the mosquitoes of Thailand (Rattanarithikul et al., 2006). The main morphological differences between three *Coquillettidia* species, *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* are described in Fig. 2.

2.3. DNA extraction, COI amplification, and DNA sequencing

After the morphological classification, two to four legs of sample mosquitoes were used to extract genomic DNA using the FavorPrep™ mini kit (Favorgen Biotech, Ping-Tung, Taiwan), according to the manufacturer's instructions. The amplification of approximately 707 base pair (bp) of the *COI* gene for DNA barcoding in this study was carried out using a pair of universal barcode primers as previously developed by Kumar et al. (2007), with the following sequences, 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′).

The PCR amplifications were performed on a thermal cycler (Biometra TOne Series, Germany) in a total reaction volume of 25 μL, containing with the following components: 4 μL of DNA template, 0.2 μM of each primer, 1× reaction buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 5% of dimethyl sulfoxide, 1.5 U Platinum Taq DNA polymerase (Invitrogen), and distilled water added up to 25 μL. The PCR program for the *COI* amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by five denaturation cycles at 94 °C for 40 s, annealing at 45 °C for 60 s, and extension at 72 °C for 1 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 54 °C for 60 s, and extension at 72 °C for 1 min, with final extension at 72 °C for 10 min, then kept at 4 °C indefinitely. Every PCR cycle included negative (without DNA) and positive (mosquito DNA from a previous study [Chaiphongpachara et al., 2022b]) controls for the amplification reactions.

PCR products of the *COI* gene were visualized using electrophoresis on 1.5% agarose gels stained with the Midori Green DNA stain (Nippon Gene, Tokyo, Japan) under the ImageQuant LAS 500 imager (GE Healthcare Japan Corp., Tokyo, Japan) to check their quality before DNA sequencing. Quality PCR products with clearly visible DNA band

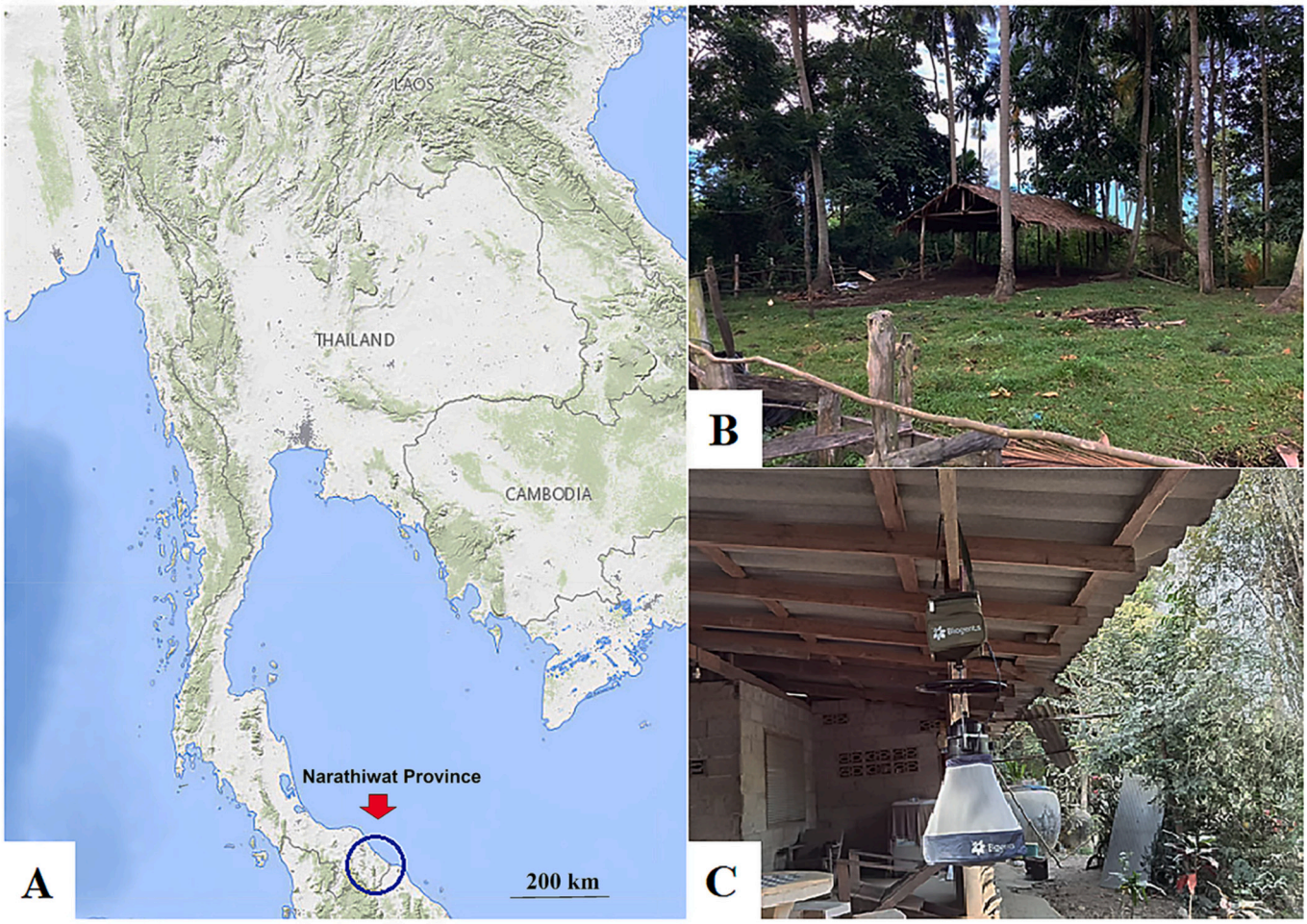

Fig. 1. Map showing Narathiwat Province, southern Thailand (A): A sample collection site in this study (B), and a BG-Pro CDC-style trap for capturing mosquitoes in the field (C). The map was retrieved from the USGS National Map Viewer, which is available for public use at the following URL: http://viewer.nationalmap.gov/viewer/.

on the agarose gel were purified and sequenced using the Sanger method in both directions, serviced by SolGent, Inc. (Daejeon, Korea).

2.4. Molecular data analysis

After DNA sequencing, trace files of partial *COI* gene sequences were manually aligned, checked, and edited to generate to create consensus sequences based on forward and reverse sequences in BioEdit software. The *COI* consensus sequences were aligned with Clustal W (Larkin et al., 2007) in MEGA X software (Kumar et al., 2018), and intraspecific and interspecific nucleotide sequence divergence were calculated based on the Kimura two-parameter (K2P) distance model. All *COI* sequences were validated for preliminary species identification by comparing their similarity with the publicly available sequences in the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) GenBank database using the Basic Local Alignment Search Tool (BLAST). A total of 36 *Coquillettidia* sequences obtained in this study were submitted to GenBank, and their accession numbers, reference sequences, and the outgroup sequence used for phylogenetic tree construction are shown in Table 1. The maximum likelihood (ML) tree of *COI* sequence dataset was constructed with the Tamura 3 parameter and Gamma distribution (T92 + G, the best-fit model) in MEGA X, with 1000 bootstrap replicates to visualize the genetic relationships among *Coquillettidia* species.

2.5. Wing geometric morphometrics

2.5.1. Slide preparation and image processing

Coquillettidia mosquitoes with complete right wings were selected, and a total of 60, 30, and 51 wings of *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea*, respectively, were used for GM analyses. To perform wing GM analyses, the right wing of each female *Coquillettidia* specimen was detached from the thorax and mounted on a microscope slide with a coverslip using Hoyer's mounting medium. The wing scales were carefully removed using a small needle under a Nikon SMZ 800 N stereomicroscope. Subsequently, *Coquillettidia* wing slides in each species were photographed using a digital camera coupled to a Nikon SMZ 800 N stereo-microscope, a scale bar of size 1 mm included on every wing image.

The GM analyses in this study used the XY Online Morphometrics online tool version 2 (Dujardin and Dujardin, 2019). In each wing image, the coordinates of 18 landmarks (Fig. 3) were digitized by a single user. The position of these anatomical landmarks was based on the several previous mosquito studies (Chaiphongpachara et al., 2022a; Saiwichai et al., 2023; Laojun et al., 2023; Sauer et al., 2020).

2.5.2. Repeatability and allometry

To assess the precision of digitized landmarks, their repeatability was tested in 10 randomly selected wings per species based on previous mosquito studies (Chaiphongpachara et al., 2022c; Saiwichai et al., 2023). Wings were digitized twice by the same user, then their

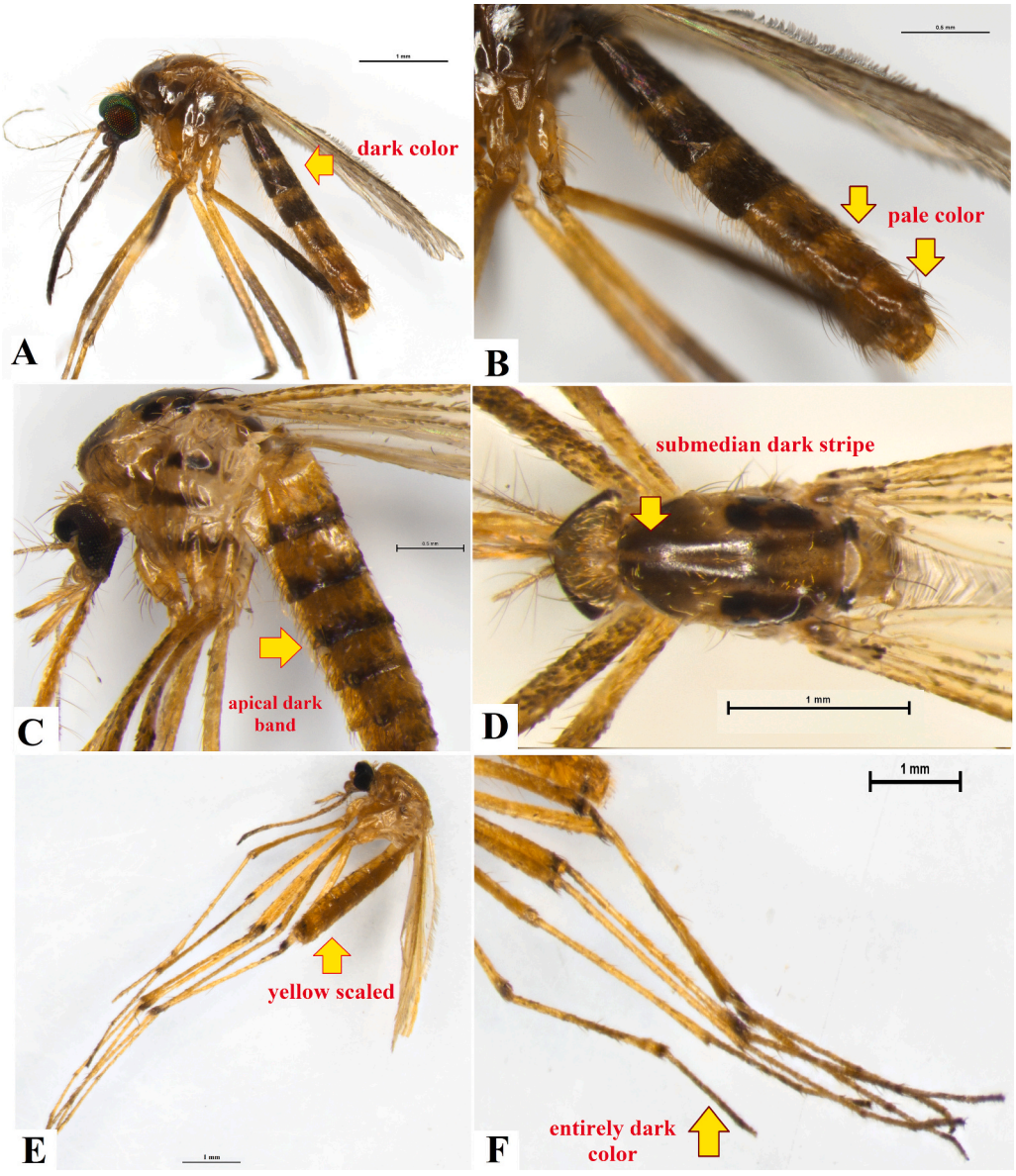

Fig. 2. Female adult of *Cq. crassipes* (A, B), *Cq. nigrosignata* (C, D), and *Cq. ochracea* (E, F). For morphologically distinctive features: *Cq. crassipes* has abdominal terga II–IV or V dark purple (A), while abdominal terga V or VI–VIII pale colour (B); *Cq. nigrosignata* has apical dark bands at abdominal terga (C) and a pair of submedian dark stripes on the anterior half at scutum (D); *Cq. ochracea* has abdominal terga II–V yellow scaled (E) and hindtarsomeres 3–5 entirely dark colour (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary GenBank accession numbers of *Coquillettidia* species, and the outgroup (*Mansonia annulifera*) used for phylogenetic analysis in this study.

Mosquito species	Country	Sequence source	GenBank accession no.
Cq. crassipes	Thailand	In this study	OP107898–909
Cq. crassipes	Singapore	GenBank	KF564771
Cq. crassipes	China	GenBank	JQ728319
Cq. nigrosignata	Thailand	In this study	OP107910–21
Cq. nigrosignata	Singapore	GenBank	KF564772, KF564775
Cq. ochracea	Thailand	In this study	OP107922–33
Cq. ochracea	South Korea	GenBank	KT358437
Cq. ochracea	Japan	GenBank	LC646365
Mansonia annulifera	Thailand	GenBank	OL743072

"repeatability" (R) index compared with those of test image sets according to Arnqvist and Mårtensson (1998). In addition, to investigate the relationship between wing size and wing shape (also known as allometry), the linear determination coefficient, r-squared (r^2), after regressing between the centroid wing size and the first principal component was performed.

2.5.3. Wing size and shape analyses

The centroid size (CS) derived from the distances between the centroid point of each configuration (wing) and each landmark (Bookstein, 1991) was used to investigate global wing size among *Coquillettidia* species. Next, quantile box plots were created to examine the variation of wing CS among *Coquillettidia* species, while statistically significant differences in CS between species were examined using nonparametric ANOVA (1000 replicates) with Bonferroni correction. The significance levels in all statistical analyses in this study were inferred at $p < 0.05$. Maximum likelihood classification based on wing CS was performed to test for the correct assignment of individuals (Dujardin et al., 2017).

For wing shape analyses, principal components (PCs) of the Procrustes residuals (also called tangent space variables) were derived from generalized Procrustes analysis and used as final shape variables in the discriminant analysis to investigate wing shape among *Coquillettidia* species, and to quantify their wing shape variations. Twenty seven PCs were used as input for the discriminant analysis. The statistically significant difference in wing shape based on the Mahalanobis distances (also called generalized distance) between *Coquillettidia* species was examined using a non- parametric permutation test (1000 replicates)

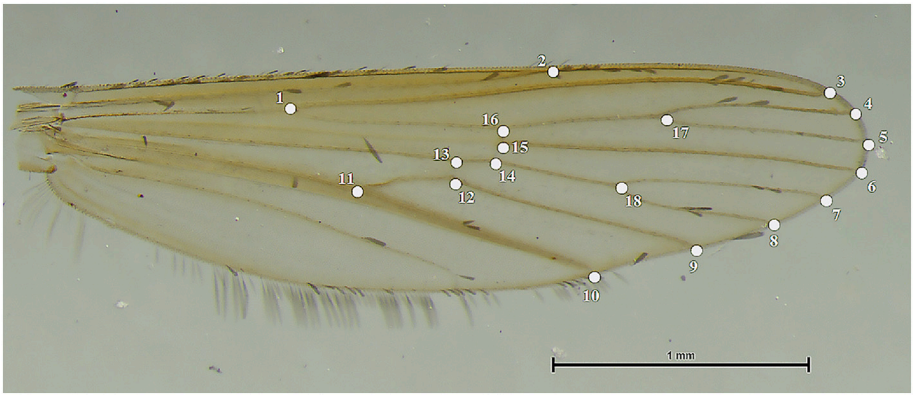

Fig. 3. Right wing of *Coquillettidia* mosquito showing 18 digitized landmarks used in geometric morphometric analysis.

with a Bonferroni correction at $p < 0.05$. A cross-validated classification procedure based on wing shape was performed to test for the correct assignment of individuals. Finally, to assess the similarity in wing shape among three *Coquillettidia* species, a hierarchical clustering tree based on Mahalanobis distances was constructed.

3. Results

A total of 419 *Coquillettidia* mosquitoes were collected from Narathiwat Province, Thailand. *Coquillettidia* specimens were morphologically classified into three distinct species, including *Cq. crassipes*, *Cq. nigrosignata,* and *Cq. ochracea*, with 197, 39, and 183 specimens, representing 47.02%, 9.30%, and 43.68% of total specimens, respectively.

3.1. Molecular analysis based on DNA barcode sequences

Twelve *Coquillettidia* mosquitoes per species were randomly selected for molecular analysis based on *COI* gene sequences. These specimens were also included in GM analyses. Results showed that *COI* sequences generated from the 36 tested *Coquillettidia* specimens had a high average AT content of 68.6%, with average nucleotide composition of A = 29.6%, T = 39%, C = 15.8%, and G = 15.6%. At the species level, all the three species harboring high AT content showed similar average nucleotide composition, with *Cq. crassipes*: A = 29.3%, T = 37.3%, C = 17.1%, and G = 16.3%, *Cq. nigrosignata*: A = 28.5%, T = 40.6%, C = 15.5%, and G = 15.4%, and *Cq. ochracea*: A = 31%, T = 39%, C = 15%, and G = 15%. The BLAST analysis in the public GenBank database revealed that *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* sequences obtained in this study shared >99% similarity, which corresponded with their morphological features based on the morphological classification.

Sequence analyses revealed an intraspecific genetic divergence of *Coquillettidia* mosquitoes that varied 0%–3.05%, with an average divergence of 0.6% (Table 2). The highest intraspecific divergence of 1.46% was observed in *Cq. crassipes*, followed by *Cq. nigrosignata* and *Cq. ochracea* with 0.29% and 0%, respectively. In contrast, the interspecific genetic divergence of *Coquillettidia* mosquitoes varied 10.10%–12.41%, with an average divergence of 11.1% (Table 2). The highest interspecific divergence of 11.84% was observed between *Cq. nigrosignata* and *Cq. crassipes*, followed by that between *Cq. ochracea* and *Cq. crassipes* and between *Cq. ochracea* and *Cq. nigrosignata* with 11.10% and 10.36%, respectively.

Table 2
Kimura two-parameter for inter- and intraspecific genetic distance between the three *Coquillettidia* species.

Coquillettidia	Average percentage genetic divergences (Min–Max)		
	Cq. crassipes	*Cq. nigrosignata*	*Cq. ochracea*
Cq. crassipes	1.46% (0.14–3.05)		
Cq. nigrosignata	11.84% (11.42–12.41)	0.29% (0.00–0.99)	
Cq. ochracea	11.10% (10.60–11.43)	10.36% (10.10–10.42)	0.00% (0.00–0.00)

Phylogenetic relationships among the three *Coquillettidia* species were analyzed using ML tree (Fig. 4). *COI* sequences from the same *Coquillettidia* species obtained in this study clustered together and were clearly separated into distinct groups with high bootstrap values (>97%), while the outgroup *Mansonia annulifera* (Theobald, 1901) sequence was distinctly separated from other *Coquillettidia* groups.

3.2. Wing geometry differences based on landmark GM analyses

3.2.1. Repeatability and allometry

A high repeatability score of 98.17% was observed in this study based on the shape of the tested image set. The allometric examination revealed a positive correlation between the wing shape and wing size ($p < 0.05$, Fig. 5), with a 70.5% allometric effect based on the linear determination coefficient (r^2) after regressing the shape on the wing size.

3.2.2. Wing size

The variation of wing CS among three *Coquillettidia* speciesis shown in Fig. 6. *Coquillettidia ochracea* had the largest wing CS of 3.89 mm ± 0.23 mm (average ± S.D.) followed by *Cq. nigrosignata* and *Cq. crassipes* with 3.79 ± 0.15 and 3.17 ± 0.20 mm, respectively (Table 3).

The mean wing CS comparison between the three *Coquillettidia* species based on a non-parametric permutation test (1000 replicates) with Bonferroni correction showed that the differences in wing size between *Cq. ochracea* and *Cq. nigrosignata* was not statistically significant ($p > 0.05$), but significant difference was observed in the comparison with *Cq. crassipes* ($p < 0.05$, Table 3). The ML classification based on wing CS displayed a total performance score of 72.34%, which ranged 46.67%–91.67% (Table 4). *Coquillettidia crassipes* yielded the highest scores with 91.67% accuracy, followed by *Cq. ochracea* and *Cq. nigrosignata* with 64.71% and 46.67% accuracy, respectively.

3.2.3. Wing shape

Superimposition of the mean landmark configurations among three *Coquillettidia* species after object alignments revealed that *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* were different in wing shape, especially in 12, 13, 17, and 18 landmark positions (Fig. 7A and B).

In addition, discriminant analysis of wing shape variation among three mosquito species showed non-overlapping species groups on the factor map, suggesting their clear differences (Fig. 8). The pairwise comparisons of wing shape based on Mahalanobis distances between the three *Coquillettidia* species showed significant differences in all species

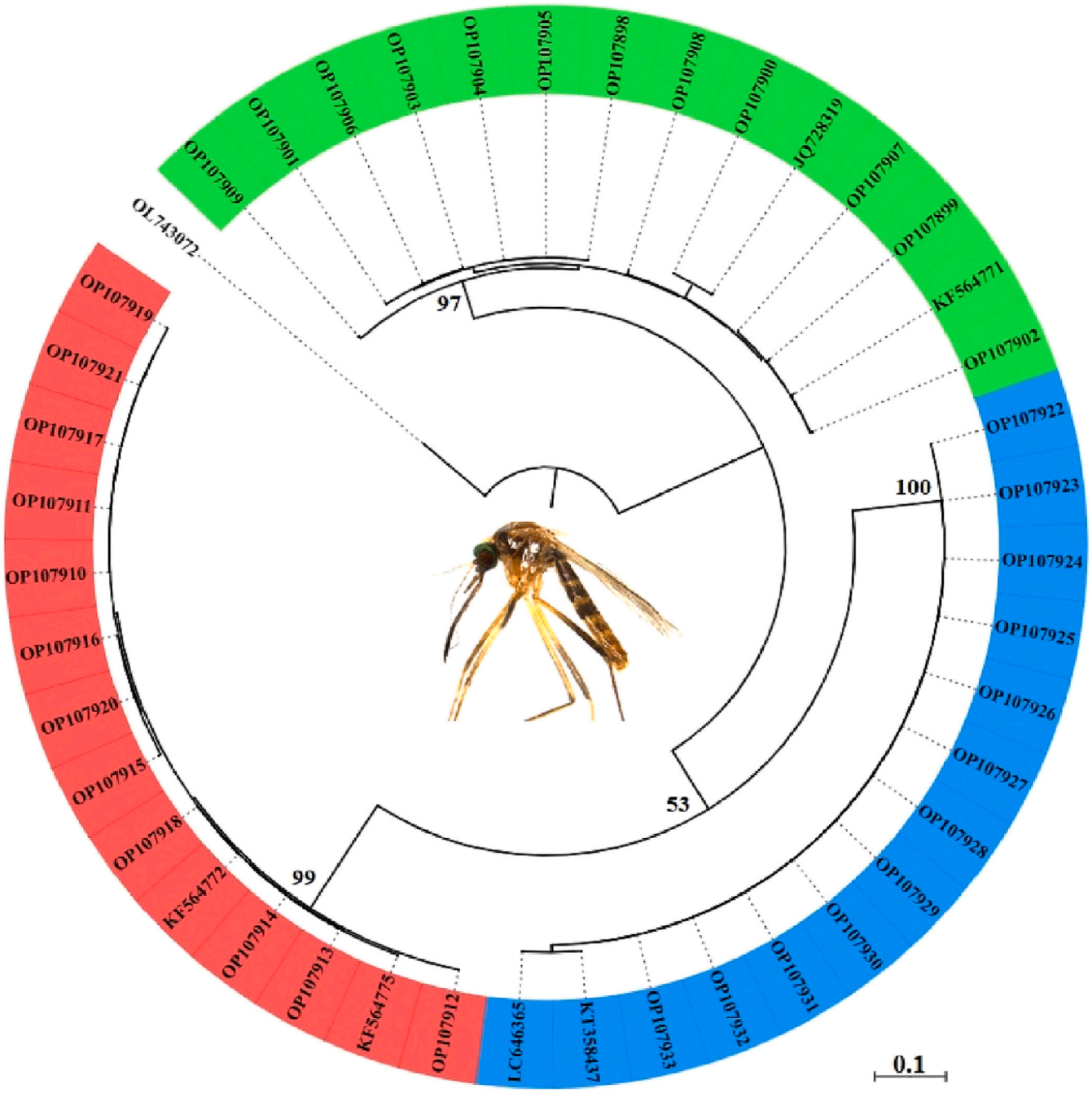

Fig. 4. Maximum likelihood (ML) tree generated with *COI* sequences of *Coquillettidia crassipes* (green), *Cq. nigrosignata* (red), and *Cq. ochracea* (blue) obtained in this study and GenBank source (these sequence data are presented in Table 1). Tree inferred using the Tamura 3 parameter with Gamma distribution (T92 + G). The bootstrap values (1000 replicates) higher than 50% are shown above the branches. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparisons ($p < 0.05$, Table 5).

The cross-validated classification results based on wing shape yielded a high total performance score of 97.16%, ranging 93.33%–100% (Table 4). *Coquillettidia crassipes* yielded the highest score of 100% accuracy, followed by *Cq. ochracea* and *Cq. nigrosignata* with accuracy scores of 96.08% and 93.33%, respectively. The hierarchical clustering tree using Mahalanobis distances revealed that *Cq. nigrosignata* was closely related to *Cq. ochracea* than *Cq. crassipes* based on wing shape features (Fig. 9).

4. Discussion

Limited information is available on *Coquillettidia* mosquitoes in Thailand and other countries; however, they are reported to be vectors of many arboviruses and *B. malayi*. Many female *Coquillettidia* species prefer to bite humans and domestic animals during nocturnal and diurnal times (Sousa et al., 2000; Maquart et al., 2021). Rattanarithikul et al. (2006) reported that all *Coquillettidia* species in Thailand could be collected using human landing catches, animal bait catches (e.g., cattle and dogs), and light traps. In the present study, both molecular and wing geometric traits were investigated to understand the differences among the three *Coquillettidia* species, including *Cq. crassipes, Cq. nigrosignata*,

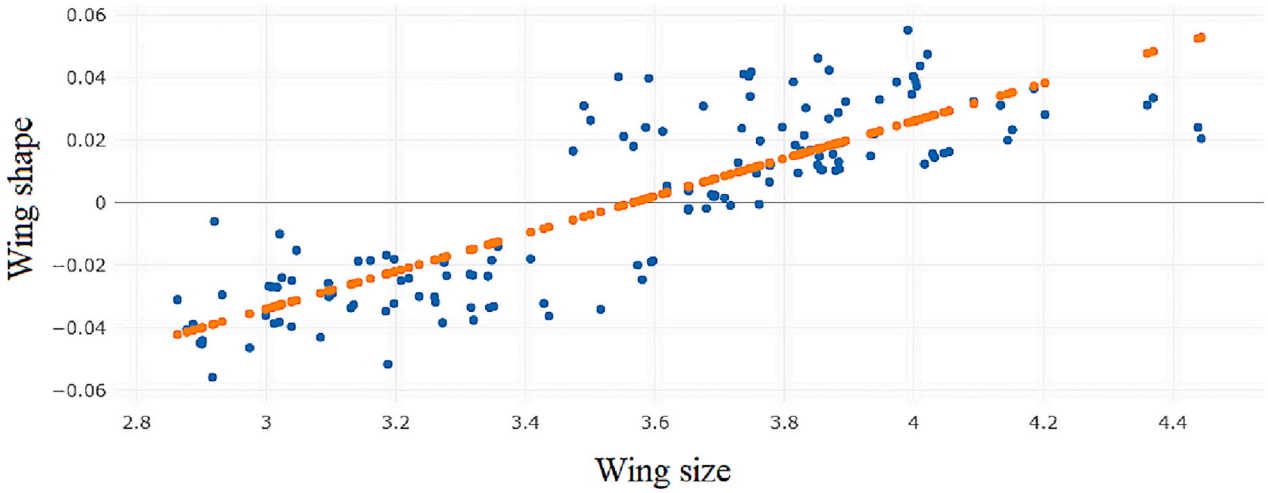

Fig. 5. Allometric plot showing the influence of wing shape (the first principal component) on wing size (wing centroid size) of *Coquillettidia* mosquitoes. The orange dotted line indicates linear regression prediction, whereas blue dot indicates individual specimen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

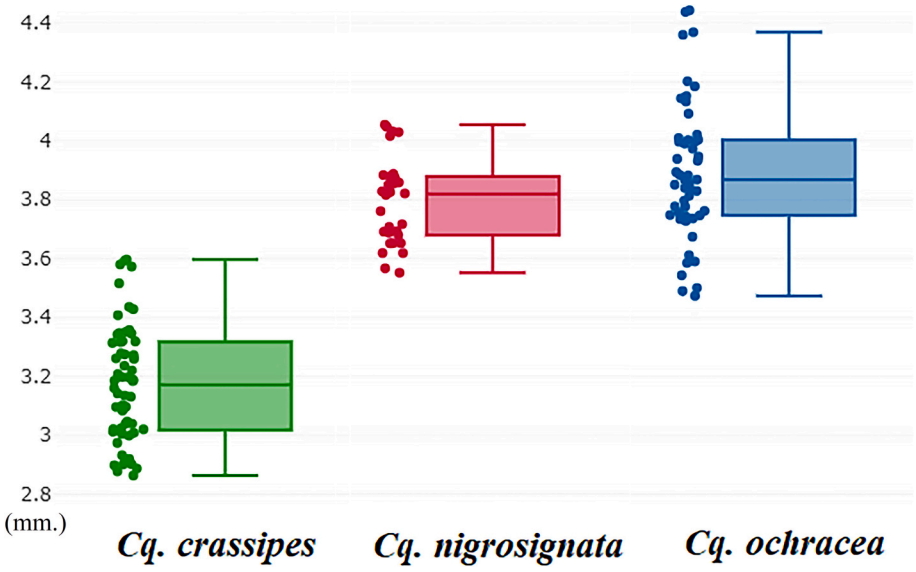

Fig. 6. Boxplot showing the variation of wing centroid size. Each box represents different *Coquillettidia* species: *Cq. crassipes* (green), *Cq. nigrosignata* (red), and *Cq. ochracea* (blue). The horizontal line within the box represent the group median, while the whiskers represent the quartiles (25th and 75th percentiles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Average wing centroid size (CS) and statistical differences among three *Coquillettidia* species.

Coquillettidia	Average wing CS (mm)	S.D.	S.E.	Min–Max
Cq. crassipes	3.17[a]	0.20	0.03	2.86–3.60
Cq. nigrosignata	3.79[b]	0.15	0.03	3.55–4.05
Cq. ochracea	3.89[b]	0.23	0.03	3.47–4.44

Different lowercase letters show significant differences in wing size among three *Coquillettidia* species at $p < 0.05$ after a Bonferroni test.

Table 4
Cross-validated reclassification values based on wing CS and wing shape of the three *Coquillettidia* species.

Coquillettidia	Percentage of reclassification (Correctly assigned/Observed individuals)	
	Based on wing CS	Based on wing shape
Cq. crassipes	91.67% (55/60)	100% (60/60)
Cq. nigrosignata	46.67% (14/30)	93.33% (28/30)
Cq. ochracea	64.71% (33/51)	96.08% (49/51)
Total performance	72.34% (102/141)	97.16% (137/141)

and *Cq. ochracea* in Thailand.

The mitochondrial *COI* gene fragments of the three *Coquillettidia* species showed the high frequencies of A+T contents of 70%, 69.1%, and 66.6% for *Cq. ochracea, Cq. nigrosignata*, and *Cq. crassipes*, respectively. This result is consistent with previous reports showing that many vector mosquitoes display high A+T contents (>60%), such as *Anopheles dirus* Peyton & Harrison, 1979, *An. baimaii* Sallum & Peyton, 2005 (Chaiphongpachara et al., 2022c), *Aedes aegypti* (Linnaeus, 1762), *Ae. albopictus* (Skuse, 1895), *Ae. scutellaris* (Walker, 1859) (Sumruayphol et al., 2016), *Mansonia dives* (Schiner, 1868), and *Ma. bonneae* Edwards, 1930 (Ruangsittichai et al., 2011). Gene sequence analyses among three *Coquillettidia* species based on K2P model showed low intraspecific

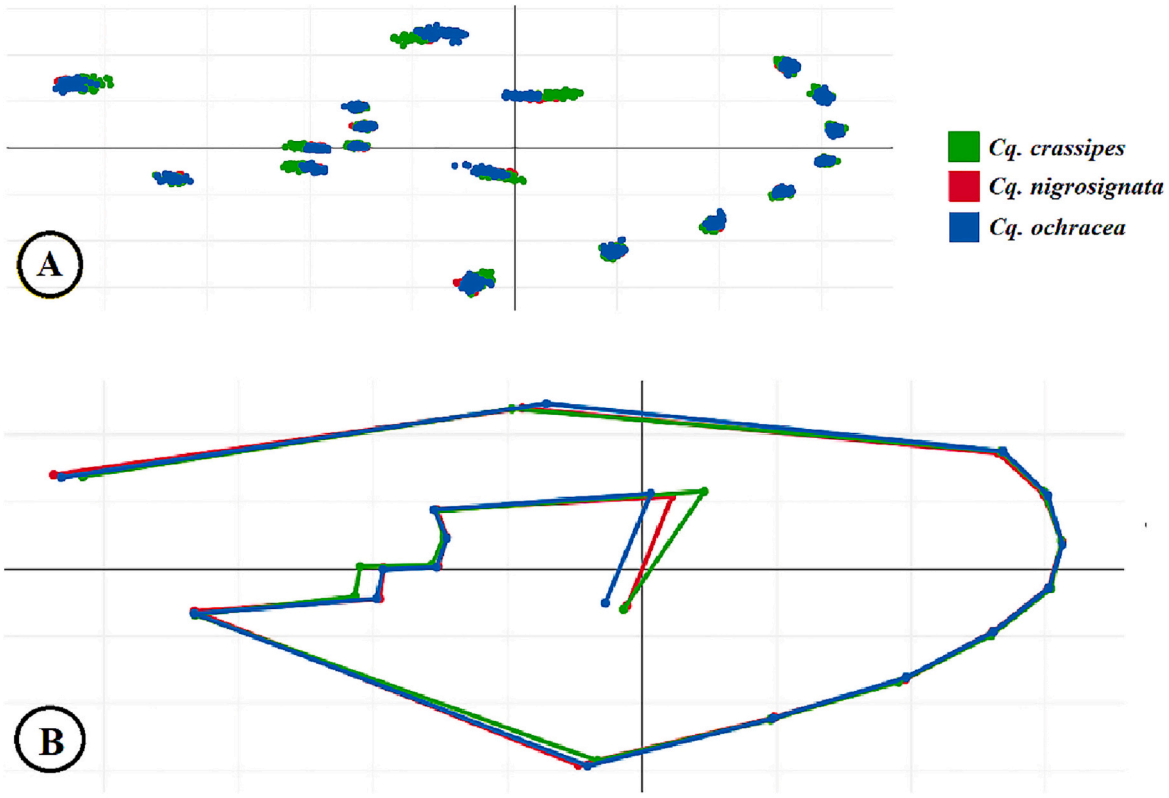

Fig. 7. Aligned objects showing the residual coordinates after Procrustes superimposition to the consensus object (A), and superimposition of the mean wing shape of the three *Coquillettidia* species (B).

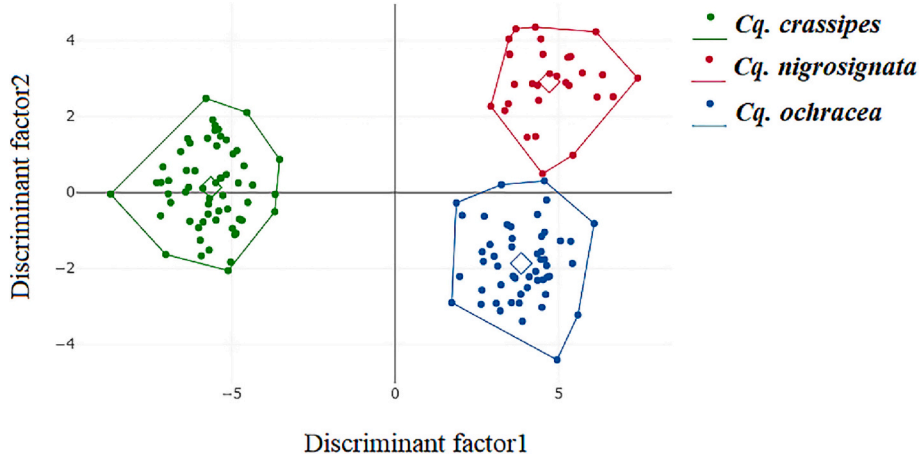

Fig. 8. Clusters obtained based on the first two discriminant factors of wing shape variables of *Coquillettidia crassipes*, *Cq. nigrosignata*, and *Cq. ochracea*. Each polygon indicates the wing shape variation of the three *Coquillettidia* species, while the dots within the polygons represent each individual sample.

Table 5
Mahalanobis distances and significant differences in wing shape of the three *Coquillettidia* species.

Coquillettidia	Pairwise Mahalanobis distance values		
	Cq. crassipes	*Cq. nigrosignata*	*Cq. ochracea*
Cq. crassipes	0.00		
Cq. nigrosignata	10.72*	0.00	
Cq. ochracea	9.70*	4.86*	0.00

Asterisks show significant differences in wing shape between *Coquillettidia* species at $p < 0.05$ after a Bonferroni test.

distances (0%–3.05%) and large interspecific distances (10.10%–12.41%). The values of intra- and inter- genetic distances of the three *Coquillettidia* species did not overlap, showing the presence of a barcoding gap, which indicated the efficiency of identification using the *COI* gene (Kress and Erickson, 2008; Madeira et al., 2021). Additionally, a phylogenetic tree based on ML analysis exhibited the three taxa including *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* based on distinct clades differentiated by branch values. Our results of genetic investigations were consistent with the results of the morphological classification. Thus, the morphological traits used to distinguish these species were valid, they correspond well to three genetically separate entities.

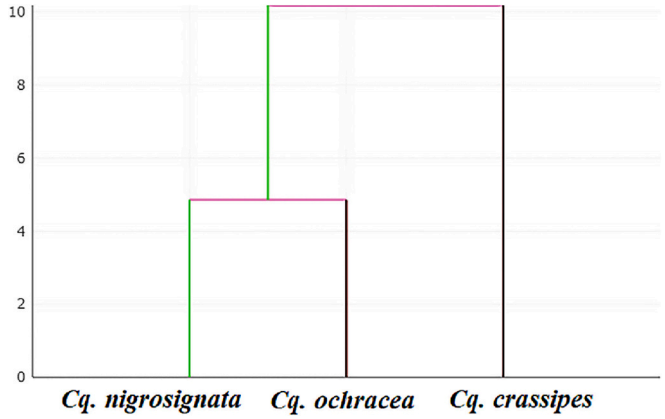

Fig. 9. Hierarchical clustering tree generated based on Mahalanobis distances showing similarity in wing shape among three *Coquillettidia* species.

No intraspecific nucleotide variation was found in the *Cq. ochracea* samples (0%), while low intraspecific variation was observed in *Cq. nigrosignata* samples (0.29%). In contrast, *Cq. crassipes* samples had intraspecific nucleotide variation of 1.46%, which was higher than those of the other two tested species. The intraspecific genetic variation reflects the demographic and evolutionary history of populations and demonstrates the capacity of species and populations to adapt to environmental changes (Harrisson et al., 2016; Walton et al., 2000). There are many reasons for low levels of intraspecific genetic variation such as the founder effects (Jamieson, 2011), the limited geographical population (Hague and Routman, 2016) and no longer exchanging genes with the parent population (Polato et al., 2017). Therefore, future studies should investigate the population structure of *Coquillettidia* species in Thailand and other countries based on several genetic markers such as the internal transcribed spacer (ITS1 and ITS2) regions of the nuclear rRNA genes, and cytochrome *b* (*Cytb*) gene to prove these ideas.

To verify the landmark digitization precision, repeatability test was performed and high values (>90%) were observed in image sets of each species. Image set inspection by repeatability test is an important step that limits measurement errors that cause misleading test results in the GM technique (Al Dujardin et al., 2010; Gómez et al., 2013). The results of allometry revealed the variation in wing shape among the three *Coquillettidia* species is under the influence of wing size variation. According to previous studies, this relationship can be found in several mosquito species such as *Culex coronator* Dyar & Knab, 1906 (Demari-Silva et al., 2014), *Cx. nigripalpus* Theobald, 1901 (De Carvalho et al., 2017), *Ae. albopictus* (Morales Vargas et al., 2013), and *An. darlingi* Root, 1926 (Motoki et al., 2012). Previous studies explained that allometry has no effect on interspecific differences, but dramatically affects intraspecific differences (Lorenz et al., 2017; Sontigun et al., 2019). Therefore, these allometric residues should be eliminated when comparing conspecific populations. In contrast, the elimination of the effect of size on shape is less justified when the populations belong to different (interspecific) species. The significant allometry (70%) in this study is due to the presence of a species (*Cq. crassipes*) that is significantly smaller than the other two (*Cq. nigrosignata* and *Cq. ochracea*). Although these two other species were almost the same size, they showed significant differences in shape, which shows that here allometry is not an obstacle to the use of the wing as a taxonomic character.

For the landmark-based GM approach, the wing size analysis based on CS revealed statistically insignificant values of 3.79 mm and 3.89 mm between *Cq. nigrosignata* and *Cq. ochracea*. This indicates that the size variable should not be used in the identification of these two species. Wing size is highly variable and sensitive to environments; thus, it is not commonly used in the taxonomy of mosquitoes (Lorenz et al., 2017). This is consistent with the cross-validated classification results based on wing CS, which indicated a total performance of 72.34%. However, the wing size of *Cq. crassipes* had clear significant differences, which could be a contributing factor in the preliminary species identification (smallest in wing size [3.17 mm]).

While wing shape contained by itself a highly significant taxonomic signal, which were supported by a high total performance score of cross-validated classification (97.16%). The superimposition of aligned mean configurations of *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* revealed differences in wing venation patterns, which confirmed the taxonomic signal of *Coquillettidia* mosquitoes in Thailand. The wing geometry showed that furcation of vein R2 + 3 of *Cq. ochracea* is far from the wing tip than *Cq. nigrosignata* and *Cq. crassipes*, respectively. In addition, the vein M1 + 2 of *Cq. crassipes* was located far from the wing tip than for those of *Cq. ochracea* and *Cq. nigrosignata*. In addition, the hierarchical clustering tree based on wing shape of *Cq. crassipes, Cq. nigrosignata,* and *Cq. ochracea* revealed consistent patterns with the phylogenetic tree constructed with *COI* gene sequences. Overall, these results in this study indicated that wing geometric information based on wing venation traits can be linked to genetic information (Lorenz et al., 2017; Gómez and Correa, 2017). However, sometimes the phenotypic differences are not correlated with the genotype (Chaiphongpachara et al., 2022c). Thus, it may be or not be linked depending on the species and environmental factors.

5. Conclusions

This study provides the first evidence of molecular and wing geometric differences to support species identification and biological variations of three *Coquillettidia* species, namely *Cq. crassipes, Cq. nigrosignata*, and *Cq. ochracea* in Thailand. In addition, the morphological classification of *Coquillettidia* mosquitoes has been completely validated by genetics based on *COI* gene. Our results derived from the molecular and morphometric differentiation of three *Coquillettidia* mosquitoes imply that our data can serve as a reference for species identification of *Coquillettidia* mosquitoes. Thus, the GM data in this study is provided in the supplemental information. However, our study did not cover *Coquillettidia* samples from across Thailand due to limitations in their distribution. It is possible that future specimen collections might find *Coquillettidia* species distributed elsewhere. Therefore, it will be important to update the data on molecular and wing geometric traits when new *Coquillettidia* reports are found, especially for *Cq. novochracea*, which was not included in this study. In addition, further studies should be conducted on the behavior and biology of these rare mosquitoes to help provide more basic information on the surveillance and prevention of mosquito-borne diseases.

Funding

This research was funded by the Suan Sunandha Rajabhat University, Thailand.

CRediT authorship contribution statement

Sedthapong Laojun: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Tanasak Changbunjong:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Suchada Sumruayphol:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Tanawat Chaiphongpachara:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Tanawat Chaiphongpachara reports administrative support was provided by Suan Sunandha Rajabhat University. Tanawat Chaiphongpachara reports a relationship with Suan Sunandha Rajabhat University that includes: employment.

Data availability

All *COI* sequences of *Cq. crassipes*, *Cq. nigrosignata*, and *Cq. ochracea* were submitted and available in the GenBank Database (https://www.ncbi.nlm.nih.gov/nuccore), accession numbers; OP107898– OP107933 (Table 1).

Acknowledgements

This study received support from Suan Sunandha Rajabhat University, Bangkok, Thailand. Additionally, we would like to thank Ms. Weaanisa Ama for *Coquillettidia* collections in Narathiwat Province, Thailand.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2023.105452.

References

Al Dujardin, J.P., Kaba, D., Henry, A.B., 2010. The exchangeability of shape. BMC Res. Notes 3, 266.
Apiwathnasorn, C., Samung, Y., Prummongkol, S., Panasoponkul, C., Loymek, S., 2009. Mosquito fauna of "Toh Daeng" swamp forest, Thailand. Southeast Asian J. Trop. Med. Public Health 40 (4), 720–726.
Arnqvist, G., Mårtensson, T., 1998. Measurement error in geometric morphometrics: empirical strategies to assess and reduce its impact on measures of shape. Acta Zool. Acad. Sci. Hung. 44 (1), 73–96.
Bookstein, F.L., 1991. Morphometric Tools for Landmark Data: Geometry and Biology. Cambrige University, Cambrige.
Bourke, B.P., Wilkerson, R.C., Linton, Y.M., 2021. Molecular species delimitation reveals high diversity in the mosquito *Anopheles tessellatus* Theobald, 1901 (Diptera, Culicidae) across its range. Acta Trop. 215, 105799.
Chaiphongpachara, T., Laojun, S., 2020. 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. Folia Parasitol. 67 (2020), 007.
Chaiphongpachara, T., Changbunjong, T., Laojun, S., 2022a. Geometric morphometric and molecular techniques for discriminating among three cryptic species of the *Anopheles barbirostris* complex (Diptera:Culicidae) in Thailand. Heliyon 8, e11261.
Chaiphongpachara, T., Changbunjong, T., Laojun, S., Nutepsu, T., Suwandittakul, N., Kuntawong, K., Sumruayphol, S., Ruangsittichai, J., 2022b. Mitochondrial DNA barcoding of mosquito species (Diptera: Culicidae) in Thailand. PLoS One 17, e0275090.
Chaiphongpachara, T., Changbunjong, T., Sumruayphol, S., Laojun, S., Suwandittakul, N., Kuntawong, K., 2022c. Geometric morphometrics versus DNA barcoding for the identification of malaria vectors *Anopheles dirus* and *An. baimaii* in the Thai - Cambodia border. Sci. Rep. 12, 13236.
Chan, A., Chiang, L., Hapuarachchi, H.C., Tan, C., Pang, S., Lee, R., Lee, K., Ng, L., 2014. DNA barcoding : complementing morphological identification of mosquito species in Singapore. Parasit. Vectors 7, 569.
Chatpiyaphat, K., Sumruayphol, S., Dujardin, J.P., Samung, Y., Phayakkaphon, A., Cui, L., Ruangsittichai, J., Sungvornyothin, S., Sattabongkot, J., Sriwichai, P., 2021. Geometric morphometrics to distinguish the cryptic species *Anopheles minimus* and *An. harrisoni* in malaria hot spot villages, western Thailand. Med. Vet. Entomol. 35, 293–301.
Chiang, G.L., Samarawickrema, W.A., Mak, J.W., Cheong, W.H., Sulaiman, I., Yap, H.H., 1986. Field and laboratory observations on *Coquillettidia crassipes* in relation to transmission of *Brugia malayi* in peninsular Malaysia. Ann. Trop. Med. Parasitol. 80 (1), 117–121.
Chonephetsarath, S., Raksakoon, C., Sumruayphol, S., Dujardin, J.P., Potiwat, R., 2021. The unequal taxonomic signal of mosquito wing cells. Insects 12 (5), 376.
Cywinska, A., Hunter, F.F., Hebert, P.D.N., 2006. Identifying Canadian mosquito species through DNA barcodes. Med. Vet. Entomol. 20, 413–424.
De Carvalho, G.C., Vendrami, D.P., Marrelli, M.T., Wilke, A.B.B., 2017. Wing variation in *Culex nigripalpus* (Diptera: Culicidae) in urban parks. Parasit. Vectors 10 (1), 423.
Demari-Silva, B., Suesdek, L., Sallum, M.A.M., Marrelli, M.T., 2014. Wing geometry of *Culex coronator* (Diptera: Culicidae) from south and Southeast Brazil. Parasit. Vectors 7, 174.
Dujardin, J.P., Dujardin, S., Kaba, D., Santillán-Guayasamín, S., Villacís, A.G., Piyaselakul, S., Sumruayphol, S., Samung, Y., Morales Vargas, R., 2017. The maximum likelihood identification method applied to insect morphometric data. Zool. Syst. 42, 46–58.
Dujardin, S., Dujardin, J.P., 2019. Geometric morphometrics in the cloud. Infect. Genet. Evol. 70, 189–196.
Feng, X., Huang, L., Lin, L., Yang, M., Ma, Y., 2017. Genetic diversity and population structure of the primary malaria vector *Anopheles sinensis* (Diptera: Culicidae) in China inferred by cox1 gene. Parasit. Vectors 10 (1), 75.
Gómez, G., Jaramillo, L., Correa, M.M., 2013. Wing geometric morphometrics and molecular assessment of members in the Albitarsis complex from Colombia. Mol. Ecol. Resour. 13, 1082–1092.
Gómez, G.F., Correa, M.M., 2017. Discrimination of Neotropical *Anopheles* species based on molecular and wing geometric morphometric traits. Infect. Genet. Evol. 54, 379–386.
Hague, M.T.J., Routman, E.J., 2016. Does population size affect genetic diversity? A test with sympatric lizard species. Heredity (Edinb). 116 (1), 92–98.
Harbach, R.E., 2023. Mosquito Taxonomic Inventory [https://mosquito-taxonomic-inventory.myspecies.info/]. Mosq. Taxon. Invent. Valid Species List.
Harrisson, K.A., Yen, J.D.L., Pavlova, A., Rourke, M.L., Gilligan, D., Ingram, B.A., Lyon, J., Tonkin, Z., Sunnucks, P., 2016. Identifying environmental correlates of intraspecific genetic variation. Heredity (Edinb). 117 (3), 155–164.
Iyengar, M.O., 1953. Filariasis in Thailand. Bull. World Health Organ. 9, 731–766.
Jamieson, I.G., 2011. Founder effects, inbreeding, and loss of genetic diversity in four avian reintroduction programs. Conserv. Biol. 25 (1), 115–123.
Joyce, A.L., Torres, M.M., Torres, R., Moreno, M., 2018. Genetic variability of the *Aedes aegypti* (Diptera: Culicidae) mosquito in El Salvador, vector of dengue, yellow fever, chikungunya and Zika. Parasit. Vectors 11 (1), 637.
Kress, W.J., Erickson, D.L., 2008. DNA barcodes: genes, genomics, and bioinformatics. Proc. Natl. Acad. Sci. 105 (8), 2761–2762.
Kumar, N.P., Rajavel, A.R., Natarajan, R., Jambulingam, P., 2007. DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). J. Med. Entomol. 44 (1), 1–7.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547–1549.
Laojun, S., Changbunjong, T., Chaiphongpachara, T., 2023. Evaluation of modern techniques for species identification of *Lutzia* mosquitoes (Diptera: Culicidae) in Thailand: geometric Morphometrics and DNA barcoding. Insects 14, 78.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., Mcgettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23 (21), 2947–2948.
Lorenz, C., Almeida, F., Almeida-Lopes, F., Louise, C., Pereira, S.N., Petersen, V., Vidal, P. O., Virginio, F., Suesdek, L., 2017. Geometric morphometrics in mosquitoes: what has been measured? Infect. Genet. Evol. 54, 205–215.
Madeira, S., Duarte, A., Boinas, F., Costa Osório, H., 2021. A DNA barcode reference library of Portuguese mosquitoes. Zoonoses Public Health 68 (8), 926–936.
Maquart, P.O., Fontenille, D., Rahola, N., Yean, S., Boyer, S., 2021. Checklist of the mosquito fauna (Diptera, Culicidae) of Cambodia. Parasite 28, 60.
Moncayo, A.C., Edman, J.D., Turell, M.J., 2000. Effect of eastern equine encephalomyelitis virus on the survival of *Aedes albopictus, Anopheles quadrimaculatus,* and *Coquillettidia perturbans* (Diptera: Culicidae). J. Med. Entomol. 37 (5), 701–706.
Morales Vargas, R.E., Phumala-Morales, N., Tsunoda, T., Apiwathnasorn, C., Dujardin, J. P., 2013. The phenetic structure of *Aedes albopictus*. Infect. Genet. Evol. 13, 242–251.
Motoki, M.T., Suesdek, L., Bergo, E.S., Sallum, M.A.M., 2012. Wing geometry of *Anopheles darlingi* root (Diptera: Culicidae) in five major Brazilian ecoregions. Infect. Genet. Evol. 12, 1246–1252.
Njabo, K.Y., Cornel, A.J., Sehgal, R.N.M., Loiseau, C., Buermann, W., Harrigan, R.J., Pollinger, J., Valkiunas, G., Smith, T.B., 2009. *Coquillettidia* (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malar. J. 8, 193.
Nugroho, S.S., Mujiyono, Ayuningrum, F.D, Setiyaningsih, R., Astuti, U.N.W., 2020. A revised checklist of mosquitoes genus *Coquillettidia* dyar, 1905 (Diptera: Culicidae) from Indonesia with key to species. Biodiversitas 21, 5772–5777.
Polato, N.R., Gray, M.M., Gill, B.A., Becker, C.G., Casner, K.L., Flecker, A.S., Kondratieff, B.C., Encalada, A.C., Poff, N.L., Funk, W.C., Zamudio, K.R., 2017. Genetic diversity and gene flow decline with elevation in montane mayflies. Heredity (Edinb). 119 (2), 107–116.
Rattanarithikul, R., Harrison, B.A., Panthusiri, P., Peyton, E.L., Coleman, R.E., 2006. Illustrated keys to the mosquitoes of Thailand: III. Genera *Aedeomyia, Ficalbia, Mimomyia, Hodgesia, Coquillettidia, Mansonia*, and *Uranotaenia*. Southeast Asian J. Trop. Med. Public Health 37 (Suppl1), 1–85.
Ruangsittichai, J., Apiwathnasorn, C., Dujardin, J.P., 2011. Interspecific and sexual shape variation in the filariasis vectors *Mansonia dives* and *Ma. bonneae*. Infect. Genet. Evol. 11, 2089–2094.
Saiwichai, T., Laojun, S., Chaiphongpachara, T., Sumruayphol, S., 2023. Species identification of the major Japanese encephalitis vectors within the *Culex vishnui* subgroup (Diptera: Culicidae) in Thailand using geometric Morphometrics and DNA barcoding. Insects 14, 131.
Sauer, F.G., Jaworski, L., Erdbeer, L., Heitmann, A., Schmidt-Chanasit, J., Kiel, E., Lühken, R., 2020. Geometric morphometric wing analysis represents a robust tool to identify female mosquitoes (Diptera: Culicidae) in Germany. Sci. Rep. 10 (1), 17613.
Sérandour, J., Ravanel, P., Tissut, M., Lempérière, G., Raveton, M., 2011. Experimental bases for a chemical control of *Coquillettidia* mosquito populations. Pestic. Biochem. Physiol. 101, 65–70.

Shepard, J.J., Andreadis, T.G., Thomas, M.C., Molaei, G., 2016. Host associations of mosquitoes at eastern equine encephalitis virus foci in Connecticut, USA. Parasit. Vectors 9 (1), 474.

Sherwood, J.A., Stehman, S.V., Howard, J.J., Oliver, J., 2020. Cases of eastern equine encephalitis in humans associated with *Aedes canadensis, Coquillettidia perturbans* and *Culiseta melanura* mosquitoes with the virus in New York state from 1971 to 2012 by analysis of aggregated published data. Epidemiol. Infect. 148, e72.

Sontigun, N., Samerjai, C., Sukontason, K., Wannasan, A., Amendt, J., Tomberlin, J.K., Sukontason, K.L., 2019. Wing morphometrics as a tool in species identification of forensically important blow flies of Thailand. Acta Trop. 190, 312–319.

Sousa, C.A., Almeida, P.G., Joao, M., Easton, P.E.R., Anselmo, M.L., 2000. On *Coquilleitidia crassipes*, a new record for Macau, with a key to adults of the subgenera and species groups of the genus. J. Am. Mosq. Control Assoc. 16 (2), 66–70.

Sumruayphol, S., Apiwathnasorn, C., Ruangsittichai, J., Sriwichai, P., Attrapadung, S., Samung, Y., Dujardin, J.P., 2016. DNA barcoding and wing morphometrics to distinguish three *Aedes* vectors in Thailand. Acta Trop. 159, 1–10.

Talaga, S., Leroy, C., Guidez, A., Dusfour, I., Girod, R., Dejean, A., Murienne, J., 2017. DNA reference libraries of French Guianese mosquitoes for barcoding and metabarcoding. PLoS One 12 (6), e0176993.

Thongsripong, P., Green, A., Kittayapong, P., Kapan, D., Wilcox, B., Bennett, S., 2013. Mosquito vector diversity across habitats in Central Thailand endemic for dengue and other arthropod-borne diseases. PLoS Negl. Trop. Dis. 7 (10), e2507.

Vythilingam, I., Sidavong, B., Chan, S.T., Phonemixay, T., Phompida, S., Jeffery, J., 2006. Species composition of mosquitoes of Attapeu Province, Lao People's Democratic Republic. J. Am. Mosq. Control Assoc. 22, 140–143.

Walton, C., Handley, J.M., Tun-Lin, W., Collins, F.H., Harbach, R.E., Baimai, V., Butlin, R.K., 2000. Population structure and population history of *Anopheles dirus* mosquitoes in Southeast Asia. Mol. Biol. Evol. 17 (6), 962–974.

Wang, G., Li, C., Guo, X., Xing, D., Dong, Y., Wang, Z., Zhang, Y., Liu, M., Zheng, Z., Zhang, H., Zhu, X., Wu, Z., Zhao, T., 2012. Identifying the main mosquito species in China based on DNA barcoding. PLoS One 7 (10), e47051.