Geometric morphometrics to distinguish the cryptic species *Anopheles minimus* and *An. harrisoni* in malaria hot spot villages, western Thailand

K. CHATPIYAPHAT1, S. SUMRUAYPHOL1, J.-P. Dujardin2, Y. SAMUNG1, A. PHAYAKKAPHON1, L. CUI3, J. RUANGSITTICHAI1, S. SUNGVORNYOTHIN1, J. SATTABONGKOT4 and P. SRIWICHAI1

1Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, 2Unité Mixte de Recherche 177-Interactions Hôte-Vector-Parasite-Environnement dans les Maladies Tropicales Négligées dues aux Trypanosomatidés, Centre International de Recherches Agronomiques pour le Développement (CIRAD), Institut de Recherches pour le Développement (IRD), Campus international de Baillarguet, Montpellier, France, 3Department of Internal Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida, U.S.A. and 4Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. *Anopheles minimus* Theobald 1901 and *An. harrisoni* Harbach & Manguin 2007 belong to the same species complex. They are morphologically similar and can exist in sympatry but have blood host preferences. The most accurate method for their identification is based on molecular techniques. Here, we measure the level of interspecific discrimination by geometric morphometry. Sixty-seven *An. minimus* and 22 *An. harrisoni* specimens were selected based on their morphological integrity and confirmed by identification polymerase chain reaction of internal transcribed spacer 2. These samples were used as reference data allowing for a morphometric identification based on geometric shape. Despite size overlap between the two species, there was a significant shape divergence allowing for differentiation of *An. minimus* and *An. harrisoni* with 90% accuracy. An intraspecific study of *An. minimus* showed a summer period associated to the reducing of wing size, which did not influence the shape-based differentiation of *An. harrisoni*. Wing venation geometry can be used to distinguish between these cryptic species mainly based on shaped divergence. This study suggests that geometric morphometrics represent a convenient low-cost method to complement morphological identification, especially concerning damaged specimens, i.e., insects having accidentally lost the anatomical features allowing a reliable morphological identification.

Key words. *Anopheles harrisoni*, *An. minimus*, geometric morphometric, species complex.

Introduction

Malaria in Thailand is concentrated along international borders, especially along the Thai–Myanmar border (Sriwichai et al., 2016). Major malaria vectors in Thailand, such as *Anopheles dirus*, *An. minimus* and *An. maculatus* all are present in different species complexes; for example, *An. minimus* complex includes other species such as *An. harrisoni* and...
An. yaeyamaensis (Taai et al., 2017). Anopheles minimus has been incriminated as the main malaria vector in the Greater Mekong Subregion, but the vector status for An. harrisoni is unclear. Because of its ability to adapt to and coevolve with vector control strategies, An. minimus is distributed throughout Southeast Asia and often occurs in sympatry with An. harrisoni (Kengluecha et al., 2005; Taai et al., 2017). Anopheles minimus is an opportunistic feeding on both humans and animals, whereas An. harrisoni is more zoophilic (Rwegoshora et al., 2002), and in Thailand, it occurs in Kanchanaburi Province (Taai et al., 2017). As these malaria vectors differ in their distribution, biting behaviour and capacity for malaria transmission, a low-cost identification method is an important tool for malaria vector surveillance to evaluate control programs. The routine of vector surveillance system requires to define and monitor the vector species, density and risk of transmission in order to develop effective response to any outbreak/recurrence of vector borne diseases.

Traditionally, mosquito species are identified external diagnostic characters (Sungvornyothin et al., 2006; Chan et al., 2014). The morphological similarity between An. minimus and An. harrisoni and their frequent sympatry may often create confusion regarding accurate identification. Misidentification of An. minimus has been reported in several past studies (Van Bortel et al., 2001; Singh et al., 2006; Singh et al., 2010) and is most likely due to the use of damaged specimens or to possible phenotypic plasticity of the species (Baba, 1950; Harrison, 1980; Yu & Li, 1984; Sucharit et al., 1988; Sucharit & Komalamisra, 1997; Jaichapor et al., 2005). Thus, molecular techniques are currently required for their accurate differentiation. Since molecular techniques are more expensive and have to be performed in well-equipped laboratories (Sumruayphol et al., 2016), there is a need for an inexpensive, fast and easy method for accurately identifying species in the An. minimus complex. Phenetic techniques such as the geometric morphometrics method have gained popularity in the past decade. This approach is based on shape (e.g., the geometry of wing venation) and, within various species complexes, it has proven effective in supplementing and enhancing morphological identification of mosquitoes (Ruangsittichai et al., 2011; Laurito et al., 2015; Sumruayphol et al., 2016; Chaipongphachara et al., 2019). This method not only can be applied to distinguish species (Lorenz et al., 2017) but also can be used to characterize inaspecific morphological variations (Dujardin, 2008). Environmental factors such as relative humidity, food abundance and larval density may produce some shape variation but seem to influence size much more than shape (Morales Vargas et al., 2010; Gómez et al., 2014). In the recent past, mosquito size was the main morphometric character studied, and consequently, mosquito size has been the topic of many discussions concerning its possible correlation with vector capacity (Dujardin, 2008; Morales Vargas et al., 2010; Gómez et al., 2014). In our study, we were more concerned with the possible interference of size variation with shape-based interspecific distinction. This study aimed to explore the power of geometric morphometric analysis of wing venation to accurately distinguish between An. minimus and An. harrisoni.

Materials and methods

Collection of mosquito samples

Adult specimens of An. minimus were collected from four villages in Komonae (N 17°31′56.9″ E 97°56′55.7″), Suan Oi (N 17°33′28.6″ E 97°55′15.7″), Tala Oka (N 17°19′23.6″ E 98°07′03.4″) and Nong Bua (N 17°20′24.8″ E 98°06′24.2″) in Tha Song Yang District (Tak Province, Thailand) where malaria is endemic (Fig. 1). The Tha Song Yang district is approximately 1920.38 km² (741.46 sq. mi) with a population of 61 161 and density of 31.8/km². The geography is mostly made up of hills and mountains, and the climate is categorized as tropical savanna climate. The villages are located near the Moei River, which separates Thailand from Myanmar, and each village consists of approximately 100 houses. The study sites include rice fields and hilly areas with agricultural plantations.

In 2015, females of An. minimus were collected near the houses along forest fringe using Centres for Disease Control and Prevention miniature light traps (BioQuip, USA: model 2836BQ) with a timer rotator (Collection Bottle Rotator model 1512.25, John W. Hock, USA), baited with CO₂ from dry ice in a bucket. The traps were set up in 30 households in each of the four villages. Mosquitoes were collected for five nights per month from 18:00 to 6:00. Monthly An. minimus group collection in 2015 from four villages was detailed in Table S1 and the climatic data of temperature, humidity and rainfall was recorded in Table S2. The season was categorized into summer (March–May), rainy (June–October) and dry cool (November–February) seasons based on rainfall, relative humidity and temperature (Table 1). To allow for an inaspecific study exploring the possible effects of the environment, especially seasons, on size and shape, adult An. minimus were randomly selected from summer (n = 87), rainy (n = 103) and dry cool (n = 111) collections (Table 1).

The mosquitoes were collected every morning, placed in a plastic Petri dish with filter paper, and labelled (e.g., trap number, type and location). The samples were stored in a Petri dish under −20°C for morphological identification. Anopheles harrisoni was harvested by simply collecting larvae from a specific breeding area with 72% humidity and a temperature of 29.9°C in Ban Pu Toei village (N 14°20′9.5″ E 98°59′20.9″ 296 m elevation), Sai Yok district (Kanchanaburi Province, Thailand). The study site, which is located in a valley between mountains with decaying plantations, consists of dense forests and slow-running streams (Fig. 1). Selection of the site was based on a recent report indicating that An. harrisoni was present in Kanchanaburi but not in Tak (Taai et al., 2017). Larvae were collected from these streams in July 2017 with a dip netting method and were transported in a plastic bottle (600 mL) to the laboratory. They were reared in plastic trays with filtered water mixed with the water from the collection site to prevent drastic changes in water conditions. The larvae were kept in an insectary (25°C, 80% relative humidity, 12 h light) and fed with ground fish food twice a day in the morning (7:30) and afternoon (15:30). Once the larvae developed to the pupal stage, they were transferred to plastic cups for adult emergence. The adult mosquitoes were fed with cotton buds soaked with
Fig. 1. Location of the mosquito collection sites in the red dots; (A) Thasongyang district, Tak province and (B) Saiyok district, Kanchanaburi province, Thailand.

10% sugar and vitamin C. They were used for morphological identification 5 days after emergence.

Selection of reference specimens

The mosquitoes selected for the interspecific study (Table 2) were those possessing an unambiguous morphological identification using the keys described earlier (Rattanarithikul et al., 2006). Currently, the main distinguishing characteristic is the presence (An. harrisoni) or the absence (An. minimus) of a humeral pale spot located at the costa of the wing (Fig. 2) (Sungvornyothin et al., 2006). The specimens having accidentally lost this diagnostic feature are said by us to be “damaged”. Thus, 67 non-damaged specimens of An. minimus were retained for the study, which covered the three seasons and the four villages (Table 2). Since they were reared and emerged in laboratory conditions, the 22 An. harrisoni specimens did not suffer any damage due to capture and transportation.

To confirm the morphological identification of the selected specimens, genomic DNA was extracted from the hind leg of the selected subset 67 adult specimens using a QIAamp® DNA Mini Kit for DNeasy Blood & Tissue Kit (Hilden, Germany). Multiplex PCR was performed using three sets of primers (ITS2A, MIA, and MIC) as described previously (Garros et al., 2004). The ITS2A serves as the universal forward primer (TGTGAACGTGACAGCACAT), whereas species were differentiated using the species-specific reverse primers for
An. minimus (CCCGTGCGACTTGACGA) and An. harrisoni (GTTCATTCAGCAACATCAGT). The PCR contained a final volume of 25 μL, which included 2.5 μL of 1× PCR buffer (Invitrogen), 0.75 μL of 50 mM MgCl₂, 0.5 μM of 10 μM deoxyribose nucleotide triphosphate, 0.3 μL of 10 μM for each primer, 0.2 units of Taq polymerase (Invitrogen), and 1 μL of DNA template. The amplification profile was one cycle at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 40 s, and an additional cycle of final extension at 72 °C for 5 min. For each reaction, 4 μL of the PCR product was subjected to 2% agarose gel electrophoresis and stained with SYBR® Safe Stain. The expected PCR product size is 310bp in An. minimus and 180 bp in An. harrisoni.

**Geometric morphometric data**

The left wings of the 89 (67 + 22) selected specimens were removed and mounted onto a slide using Hoyer medium (Schauff, 2001) for imaging and landmark digitization. The wing images were taken using the NES-Elements D 3.2 software and a manual microscope (Nikon Eclipse 6600, Japan) connected with a digital camera (Nikon Digital Sight DS-Ri1, Japan). Sixteen type I landmarks (Bookstein, 1991) were used (Fig. 3) according to previous descriptions (Sumruayphol et al., 2016). The scale unit of each wing image was 1 mm.

**Statistical analysis**

The CLIC package version 99 was used for statistical analysis (https://xyom-clic.eu/the-clic-package/). Wing metric data were collected as x and y coordinates and transformed using generalized Procrustes analysis (i.e., they were corrected for size to focus on shape and for position and orientation to remove non-biological changes). The resulting shape variables were called partial warps (PW); the final shape variables were represented by relative warps (RW), which are the principal components of the PW (Rohlf & Slice, 1990).

The global size of the wing was computed as centroid size (Bookstein, 1991), and the units of centroid size, originally pixels, were converted to millimetres (Ruangsittichai et al., 2011). Size and shape variables were analysed separately, and their possible relationship, or allometry, was measured by linear regression techniques. The statistical significance of size difference between groups was estimated using a non-parametric test with 1000 between-group permutations, and pairwise post hoc analysis with the P value of 0.05.

The discriminant analysis (DA) used to separate species was performed using the set of the first RW adapted to the sample size as input. The DA was illustrated by a histogram showing species classification along with the unique discriminant factor. The Mahalanobis distance was used as a measure of shape divergence between groups, and statistical significance was based on permutation tests (1000 runs) with the P value of 0.05. Cross-check (also called validated) classification scores based on shape variation (thus, excluding size variation) were calculated using the jackknife procedure (Manly, 2004).

To evaluate possible allometric effects on shape variation (i.e., to determine to what extent changes in size could contribute to the shape divergence observed between species), the linear correlation coefficient was calculated between size and the unique discriminant factor derived from shape variation (Dujardin, 2008).

To ensure that digitization was consistent throughout the study, the 67 An. minimus and 22 An. harrisoni wings were digitized twice. A repeatability test was then performed as an indirect estimate of the error in measuring the wing size.
Anopheles minimus geometric morphometrics  

Fig. 2. The morphological character used to distinguish between An. minimus (A) and An. harrisoni (B) is the presence of a humeral pale spot (HP) that is only present in An. harrisoni (arrow). The specimens having accidentally lost this small area of the wing are said by us to be “damaged”.

Fig. 3. The 16 anatomical landmarks selected for the geometric morphometric analyses. The unit of the wing scale is in millimetres.

and shape (Arnqvist & Märtensson, 1998). A global estimator of repeatability for shape was obtained using the Procrustes ANOVA (Klingenberg & McIntyre, 1998).

Results

Selection of reference material

Morphological identification of females of An. minimus was based on the lack of the humeral pale spot (Fig. 2), and all originated from Tak Province, whereas An. harrisoni specimens from Kanchanaburi, unequivocally possessed the humeral pale spot present. Multiplex PCR based on ITS2 amplification confirmed that all 67 samples collected in Tak Province were An. minimus (product size 310 bp), whereas all 22 samples collected in Kanchanaburi were An. harrisoni (product size 180 bp) (Garros et al., 2004).

Morphometric characterization of species

The repeatability score of An. minimus was 99.56% for centroid size and 93.17% for shape. The repeatability score of An. harrisoni was 99.57% for centroid size and 88.49% for shape. The mean centroid size of An. minimus (2.73 mm) was significantly smaller than that for An. harrisoni (2.89 mm); however, the range of size variation in the two species significantly overlapped (Fig. 3A). The DA showed an almost complete and significant separation of shape between An. minimus and An. harrisoni (Fig. 4). The discriminant analysis showed an almost complete and significant separation of shape between An. minimus and An. harrisoni (Fig. 5). The total cross-check classification score of shape-based species diagnostic was 90% (80/89), 88% (59/67) for An. minimus and 95% (21/22) for An. harrisoni. The influence of size variation on this shape-based diagnostic was 9% (Fig. 6).

Geographic and climatic subpopulations of Anopheles minimus

The mean centroid size of An. minimus for the summer was 2.62 mm, significantly smaller than the 3.19 mm value observed in the rainy and cool dry seasons (Fig. 3B). The wing shape showed significant differences between seasons with p-values ranging from 0.001 to 0.023. Despite this significant shape variation, it was not possible to accurately classify the wings according to the seasons and the classification score ranged between 37% and 54%.

Discussion

Anopheles minimus and An. harrisoni are two cryptic species often found in the same locations in western Thailand (Sungvornyothin et al., 2006). The unambiguous morphological distinction between these species requires non-damaged specimens; therefore, molecular techniques may be necessary in case of doubt.

PCR-based techniques commonly represent the most reliable diagnostic tools. Since more than one genetic marker may be needed to differentiate multiple species in a complex, the multiplex PCR technique may be required (Garros et al., 2004). For instance, the D3 domain of the 28S rDNA failed to distinguish An. harrisoni from An. fluvialis (Singh et al., 2006), although this marker is the primary marker to discriminate between An. minimus and An. harrisoni (Sharpe et al., 1999; Harbach, 2019). Currently, multiple molecular markers from both ribosomal genes and mitochondrial DNA are available to distinguish An. minimus from An. harrisoni (Harbach et al., 2007).
Our molecular analyses confirmed the validity of the diagnostic character described by Rattanarithikul et al. (2006) (i.e., a humeral pale spot located at the costa of the wing). However, this small tuft of white scales is labile and can easily be lost in damaged specimens, making it difficult or impossible to recognize the species. Here, we selected two samples of unquestionably confirmed specimens of An. minimus and An. harrisoni and used them as reference data to evaluate the diagnostic power of the geometric technique approach.

The morphometric method applies after a wing preparation procedure involving slide mounting and imaging preparation.

Skills in these aspects are common among entomologists and do not represent procedural issues (Sumruayphol et al., 2016; Chaiphongpachara et al., 2019).
The two cryptic species, *An. minimus* and *An. harrisoni*, had overlapping sizes but possessed divergent shapes regarding their wing venation. Our study demonstrated that this feature could be used to distinguish between them. This inexpensive and simple method represents an effective technique to confirm throughout the year the identity of damaged specimens, i.e., insects having accidentally lost the anatomical features allowing a reliable morphological identification.

**Abbreviations**

GPA generalized Procrustes analysis  
DA discriminant analysis  
RW the relative warps  
PW the partial warps  
CS centroid size  
HP humeral pale spot  
DF discrimination factors  
ITS2 internal transcribed spacer 2  
PCR polymerase chain reaction  
MgCl₂ magnesium chloride  
dNTP deoxyribose nucleotide triphosphate  
μL microliter  
μM micromolar  
°C degree Celsius  
ml milliliter  
mm millimetre  
h hour  
bp base pair

**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1**: Monthly *An. minimus* group collection in 2015 from four villages in Tha Song Yang district, Tak province.

**Table S2**: Climatic data of temperature, humidity, and rain fall in 2015 in Tha Song Yang district, Tak province.

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The authors declare that they have no conflict of interests.

**Ethics approval and consent to participate**

The study was approved by the Faculty of Tropical Medicine-Institute Animal Care and Use Committee (FTM-ACUC 026/2018) Mahidol University, Thailand.

**Author contributions**

PS, JPD, LC, and JS contributed to overall study design. KC, SS, YS, AP, and JR identified the study location, mosquito...
collection, and confirmation of vector species. KC, SS, PS, and JPD analysed the data. KC, PS, SS, JPD, JS, and LC drafted and revised the manuscript. All authors read and approved the final manuscript.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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