Ticks and Tick-borne Diseases 10 (2019) 495–503

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Geometric morphometrics of the scutum for differentiation of trombiculid mites within the genus *Walchia* (Acariformes: Prostigmata: Trombiculidae), a probable vector of scrub typhus

Sungsit Sungvornyothin[a], Rawadee Kumlert[a,b], Daniel H. Paris[c,d,e], Anchana Prasartvit[f], Piengchan Sonthayanon[c,g], Chamnarn Apiwathnasorn[a], Serge Morand[h], Alexandr A. Stekolnikov[i], Suchada Sumruayphol[a,*]

[a] *Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*
[b] *The Office of Disease Prevention and Control 12, Songkhla Province, Department of Disease Control, Ministry of Public Health, Thailand*
[c] *Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*
[d] *Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland*
[e] *Faculty of Medicine, University Basel, Basel, Switzerland*
[f] *Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand*
[g] *Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*
[h] *Centre d'Infectiologie Christophe Merieux Laos, Samsenthai Road, Kaoyot Village, Sisathanak District, Vientiane Capital, Laos*
[i] *Zoological Institute of the Russian Academy of Sciences, Universitetskaya Embankment 1, Saint Petersburg, Russia*

ARTICLE INFO

Keywords:
Landmark-based geometric morphometrics
Outline-based geometric morphometrics
scutum
Walchia
Trombiculid mites
Chigger
Scrub typhus
Orientia tsutsugamushi

ABSTRACT

The vectors of scrub typhus are the larval stage of trombiculid mites, termed "chiggers". These vectors are very small – the larvae are approximately 0.2 mm in size - and therefore their morphological identification is difficult. Trombiculid mites are widely distributed across Asia and they can be identified at the genus level by the shape, size and setae/sensilla distribution of their dorsal chitin plate (scutum = shield), while morphological identification at the species level requires more mite characteristics.

We recently developed a methodology to ascertain paired matched genotype and morphotype of individual chiggers, based on autofluorescence and brightfield microscopy with subsequent molecular identification using the *COI* gene (approximately 640bp length). However, based on 20 chigger specimens characterised by paired genotypic and morphological data consisting of the four species [*Walchia ewingi* with 2 subspecies]: *Walchia ewingi lupella* (n = 9), *W. ewingi ewingi* (n = 2), *W. alpestris* (n = 2), *W. kritochaeta* (n = 5) and *W. minuscuta* (n = 2) we found evidence of genetic polymorphism and morphological plasticity within the genus *Walchia*. The phylogenetic inference of the intra-genus relationships within the *Walchia* spp., based on *COI* gene (*Blankaartia* spp. served as outgroup), delineated the five included species by an average interspecific divergence of mean distance 0.218 (0.126 – 0.323).

We therefore applied landmark-based and outline-based geometric morphometric (GM) approaches to differentiate *Walchia* species using scutum measurements. A total of 261 scutum images of *Walchia* spp. were examined by landmark-based GM (140 chigger specimens) and outline-based GM (121 specimens) techniques. All *Walchia* spp. showed significant differences in scutum size and shape. *W. minuscuta* showed the smallest mean scutum size in both techniques. The largest scutum was found in *W. ewingi lupella* and *W. ewingi ewingi* by landmark-based and outline-based GM analysis, respectively. The scutum shapes of *W. alpestris* and *W. minuscuta* were clearly distinguished from the other species. Cross-validated classification scores were different depending on species and digitizing techniques and landmark-based GM showed better scores than outline-based GM. We conclude that the morphologically closely-related trombiculid mite species can be further differentiated by their scutum features alone, using GM approaches. This technique is a promising tool for the much-needed characterization studies of chiggers and needs evaluation using matched morphometric and genotyping data for other genera of trombiculids.

---

[*] Corresponding author at: Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, 10400 Bangkok, Thailand.
E-mail address: suchada.sum@mahidol.ac.th (S. Sumruayphol).

https://doi.org/10.1016/j.ttbdis.2018.11.013
Received 21 April 2018; Received in revised form 1 November 2018; Accepted 14 November 2018

1. Introduction

Scrub typhus is a mite-borne infectious disease transmitted by the larval stage of trombiculid mites called “chiggers” and is widely distributed in the Asia-Pacific region, especially in agricultural areas. Scrub typhus is a major cause of febrile illness, mainly in the Northern region of Thailand, and with a seasonal peak occurrence around the rainy season. Regional morbidity rates were highest in the North (36.33), followed by the South (9.48), Northeast (7.00) and Central part (0.65) of Thailand respectively (Bureau of Epidemiology MoPH Thailand, 1980). Chiggers are vectors of *Orientia tsutsugamushi* the causative pathogen of scrub typhus, while the adult stage of trombiculid mites represents a reservoir host (via transovarial transmission). The occurrence of chiggers relates to rodent and human habitats, which is why ecological, entomological and reservoir host investigations are important for surveillance strategies and documenting the eco-epidemiology of scrub typhus.

The proved vectors of scrub typhus are different species of the genus *Leptotrombidium*, but species of the genera *Ascoschoengastia, Neotrombicula*, and representatives of the subfamily Gahrliepiinae, including *Walchia*, could serve as intrazootic vectors, maintaining the disease cycle in animals (Traub and Wisseman, 1974). The pathogen of scrub typhus was extracted from some species of *Walchia* (Liu et al., 1999; Yu and Lin, 1957), although their ability to attack humans and transmit *Rickettsia* is not evident.

Trombiculid mites are identified by microscopic examination of their morphological characteristics. The single most important feature for identification of chiggers is the central dorsal shield called scutum (Latin for “shield”). Its shape, size, distribution of attached setae (fine hairs) and sensilla (sensory hairs) allow identification of mites to the genus level (Nadchatram and Dohany, 1974). Additional characteristics are required for identification to the species level; these include the shape and arrangement of body setae (chaetotaxy), and features of gnathosoma, legs and coxae (Vercammen-Grandjean and Langston, 1976).

We recently described a novel method for microscopical examination whereby the mite specimen remains intact and available for subsequent DNA extraction – making the availability of paired matched morpho-genotyping possible (Kumlert et al., 2018). Chiggers of the *Walchia* genus (family Trombiculidae) are very common in Southeast Asia and often found in the ears and genital areas of rodent hosts (Chaisiri, 2016). However, within the genus *Walchia* there was evidence of genetic polymorphism and morphological plasticity, which translated into difficulties for morphological identification at the species level. Morphological identification of chigger species can be complicated further if specimens are damaged or badly fixed – as this mostly affects legs and mouth parts, which are important morphological criteria, an approach based on the usually intact scutum is most promising.

New methods and alternative techniques are increasingly used in other fields of entomology like DNA barcoding and morphometrics (Dujardin, 2008; Dujardin et al., 2014). Geometric morphometrics is an attractive technique for rapid low-cost identification of vectors previously used for characterizing medically important insects and ticks (De La Riva et al., 2001; Josek et al., 2017; Pretorius and Clarke, 2001; Ruangsittichai et al., 2011; Voltzit and Pavlinov, 1996). Morphometrics based on wing shape variations are used for comparisons between laboratory and field specimens of mosquitoes, sandflies or *Stomoxys* flies derived from different geographical locations (Changbunjong et al., 2016; Dujardin and Le Pont, 2000; Jaramillo-O et al., 2015; Prudhomme et al., 2016; Vargas et al., 2013). This technique successfully distinguished *Aedes albopictus* from morphologically similar species such as *Ae. flavopictus or Ae. riversi* (Dujardin et al., 2014) and *Stomoxys pullus* from close species like *S. uruma* (Changbunjong et al., 2016). Given the potential of geometric morphometric analyses in sandflies, *Aedes*, *Anopheles* spp. and *Stomoxys* flies (Changbunjong et al., 2016; Dujardin and Le Pont, 2000; Jaramillo-O et al., 2015; Sumruayphol et al., 2016), we opted for a geometric morphometric approach to differentiate between *Walchia* spp. using only the scutum.

In this study, we hypothesized that scutum-based geometric morphometrics provide congruent results with *COI* sequence analysis and agree with complete morphological ID based on current taxonomic charts. Hence, we attempted to classify the disparate species of the genus *Walchia* by landmark-based and outline-based geometric morphometrics of the scutum, and evaluated this method as possible complimentary or alternative approach to the *COI* DNA sequence genotyping and classical morphotyping.

2. Materials and methods

2.1. Collection and preparation of chigger mites

Chigger specimens were collected from Vientiane Province, Laos PDR. The study was approved by the National Ethics Committee for Health Research (Lao PDR, No 51/NECHR, 2016) and Mahidol University-Institute Animal Care and Use Committee (MU-IACUC) (MU-IACUC 2016/024). Animals were treated in accordance with the guidelines of the American Society of Mammalogists, and with the European Union legislation (Directive 86/609/EEC) and trapped as previously described (Kumlert et al., 2018).

A total of 140 and 121 trombiculids were selected for landmark-based and outline-based geometric morphometrics respectively in this study (Table 1), of which 140 *Walchia* spp. were identified by standard morphotyping based on classic pictorial keys (Nadchatram and Dohany, 1974; Traub and Evans, 1957). Chigger specimens were mounted on permanent slides using Nesbitt's fluid or on temporary slides using ethanol for either fluorescent or brightfield microscopy for morphological identification as previously described (Kumlert et al., 2018).

2.2. Morphological identification

Recently, we developed an approach based on autofluorescence (AF) and brightfield (BF) microscopy describing these procedures in detail (Kumlert et al., 2018). All images were obtained using a fluorescein isothiocyanate (FITC) filter with an epifluorescence illuminator (mercury or xenon lamps) and/or white light (halogen lamp) in brightfield mode, as previously described (Kumlert et al., 2018). The Nikon FITC filter used corresponded to excitation wavelengths 467–498 nm and emission wavelengths 513–556 nm as detailed by the manufacturer.

Identification to the genus level followed the keys published by Fernandes and Kulkarni (2003); Nadchatram and Dohany (1974) and Vercammen-Grandjean (1968). Identification to the species level followed the published identification keys by Womersley (1952); Fernandes and Kulkarni (2003) and Traub and Evans (1957). Detailed AF images of the scutum were acquired for each species of *Walchia* and saved as GIF files for subsequent measurements. A total of 16 features were imaged in detail prior to DNA extraction, to enable second opinions and the retrospective confirmation of the exact morphotype, after the mite specimen was destroyed.

2.3. Molecular identification

DNA of individual chigger mites was extracted as previously described (Kumlert et al., 2018). Briefly, the QIAGEN Blood & Tissue kit (QIAGEN Sciences, MD, USA) was used, ethanol was thoroughly removed and chiggers were digested in tissue lysis buffer after incision with a 30 G needle under an inverted microscope. DNA quantity and quality was assessed and served as template for the mitochondrial cytochrome c oxidase subunit I gene (*COI*) based PCR assay (Vrijenhoek, 1994). A phylogenetic tree was constructed using CLC Workbench v7.7.1 (QIAGEN, Germany) and MEGA v6 software (Tamura et al.,

Table 1
Overview of chiggers characterized as *Walchia* species by morphotyping and *COI* gene genotyping used for analysis.

Rodent species *(N captured)*	Rodents with *Walchia* spp.	Chigger taxa	Successfully morphotyped (Landmark)	Successfully morphotyped (Outline)	Successfully genotyped
Bandicota indica (n = 16)	7/16 (44%)	*Walchia ewingi ewingi*	12	10	1
		Walchia ewingi lupella	41	37	9
		Walchia kritochaeta	1	1	0
Berylmys berdmorei (n = 1)	0 (0%)		0	0	0
Berylmys bowersi (n = 1)	1/1 (100%)	*Walchia alpestris*	15	15	2
Maxomys surifer (n = 3)	3/3 (100%)	*Walchia kritochaeta*	28	22	4
		Walchia minuscuta	5	4	1
Mus caroli (n = 9)	3/9 (33%)	*Walchia kritochaeta*	1		1
		Walchia minuscuta	13	10	0
Niviventer sp. (n = 4)	2/4 (50%)	*Walchia kritochaeta*	2	1	0
		Walchia minuscuta	3	3	1
Rattus exulans (n = 20)	0/20 (0%)		0	0	0
Rattus sakaretensis (n = 10)	2/10 (20%)	*Walchia ewingi ewingi*	6	6	1
		Walchia ewingi lupella	2	2	0
		Walchia minuscuta	2	2	0
Rattus tanezumi (n = 8)	4/8 (50%)	*Walchia ewingi lupella*	2	2	0
		Walchia kritochaeta	1	2	0
		Walchia minuscuta	6	4	0
Total	**22/72 (31%)**		**140**	**121**	**20**

2013). Intra- and interspecific variation was analyzed by computed pairwise distances and comparisons between group mean distances. A total of 20 chigger mites representing the following four species [*Walchia ewingi* with 2 subspecies] were selected: *W. ewingi lupella* (n = 9), *W. ewingi ewingi* (n = 2), *W. alpestris* (n = 2), *W. kritochaeta* (n = 5) and *W. minuscuta* (n = 2) (Table 1).

2.4. Geometric morphometric analyses

2.4.1. Landmark-based GM method

Landmark-based geometric morphometrics use coordinates of reliable “landmarks” and the spatial information they share; for chiggers the anatomical loci representing insertion sites of sensilla and setae on the scutum are arguably homologous among all individual chiggers of the same genus in the analysis. The landmark-based GM approach was based on the difference in setae and sensilla distribution (insertion sites) of the scutum. These are easily visible even if the setae or sensilla have been damaged or removed. The recently described autofluorescent properties of the scutum identified 6 distinct fluorescent insertions of setae and sensilla, which served as representative, specific and readily identifiable morphological features among individual chiggers (Kumlert et al., 2018). These insertions were chosen as landmarks and were digitized in the same order for each specimen. A total of 6 landmarks were digitized for the scutum of *Walchia* spp. (Fig. 1A).

The centroid size (CS) was computed for scutum size estimation. CS is calculated from the square root of the sum of the squared distances between the center of the configuration of landmarks and each individual landmark (Efron, 1983). Statistically significant difference of mean CS among species were evaluated by non-parametric tests (at 1000 cycles) with pair wise post hoc analysis (Bonferroni correction), $p < 0.05$ was considered statistically significant.

In order to compare the shapes derived from different landmark configurations, the raw coordinates were adjusted for proper superimposing, which included scaling for size. This procedure termed “Procrustes superimposition” produces “shape variables”, which are normalised via rotational fit projection techniques to “partial warps” which can be used for standard statistical multivariate analyses (Dujardin, 2008; Rohlf and Slice, 1990). Partial warp (PW) scores were used as shape variables, which were then analysed by principal component analysis (PCA).

Each scutum landmark was plotted in relation to the distribution of the other points to estimate the difference between species providing the “generalized” or Mahalanobis distances between the landmark data for each individual chigger in the studied population. Then, a NJ (Neighbor joining) tree was constructed based on Procrustes distances. Statistically significant difference of mean scutum shape among species were analysed by non-parametric tests (at 1000 cycles, with pair wise post hoc analysis (Bonferroni correction), $p < 0.05$ was considered statistically significant).

2.4.2. Outline-based GM method

Outline-based GM analysis is based on coordinates of the outline derived from the unique shape of the scutum, which already serves for differentiation of trombiculid mites to the genus level, as the size and shape of the scutum differ between the genera. Hence, we defined the outline of the *Walchia* species scutum as the external contour (Fig. 1B). The scutum outlines were digitised for 121 *Walchia* specimens, the perimeter coordinates of the contour outline were calculated and the scutum shape variables were computed using the elliptic fourier analysis (EFA). The contour outline is documented as sine and cosine curves of which the successive frequencies (harmonics) are measured, as described previously (Dujardin, 2008; Kuhl and Giardina, 1982). Similarly, a NJ tree was constructed based on the Procrustes distances for outline-based GM species differentiation (at 1000 cycles, with pair wise post hoc analysis (Bonferroni correction), $p < 0.05$ was considered statistically significant).

2.4.3. Assigning individuals to species

Discriminant analysis (DA) of shape variables was analysed for classification to species of each individual. DA was plotted by factor maps to show species classification. A cross-validated classification procedure was applied where each sample is assigned to its closest group without being used to help determine a group center (Dujardin et al., 2010; Manly, 2004).

2.5. Morphometric software

All analyses of the morphometric data were performed using various module in CLIC package version 97 (freely available at https://xyom-clic.eu) (Dujardin et al., 2010; Dujardin and Slice, 2007) for geometric and multivariate analyses. COO (“**C**ollection of **Coo**rdinates”) module used for digitize landmark and outline of the scutum. MOG (“**MO**rfometria **G**eometrica”) (landmark) and FOG (“**F**ourier **O**utlines **G**raphics”) (outline) modules were used to produce size and shape variables, perform PCA and DAs, evaluate Procrustes distances, construct box plots. VAR (“**V**ariation **a**nd va**r**iance”) and PAD (“**P**ermutaciones,

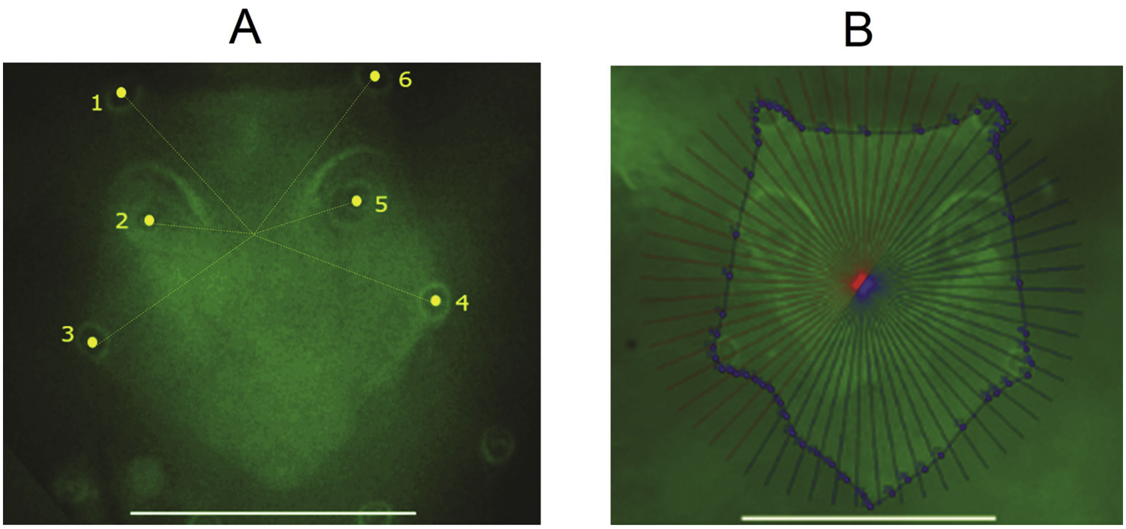

Fig. 1. Two methods to estimate the CS by landmark-based GM and perimeter by outline-based GM analyses.
The positioning of 6 “landmarks” based on the insertions of setae and sensilla is shown in panel A - these data are digitized on the scutum of a Trombiculid mite of the genus *Walchia* for landmark-based GM analysis. Multiple outline datapoints are digitized for the outline-based GM analysis of the chigger scutum of the *Walchia* spp. – depicted in panel B. (AF, magnification 400x, scale bar 35 μm).

Analisis **D**iscriminante”) modules were used to test statistical significance of differences in the size and shape variables among *Walchia* species. PAD module was used for analyzing a validated classification.

3. Results

3.1. Scutum size variation

Centroid size (CS) was used exclusively for landmark-based GM analysis. Mean scutum CS comparison of the 4 species within *Walchia* derived from landmark-based GM analysis showed size variation (Fig. 2A, Table 2). *W. ewingi lupella* had the largest scutum CS [mean (min-max) 0.0553 mm (0.0513-0.0593)]. Whereas, *W. minuscuta* had the smallest CS [mean (min-max) 0.0415 mm (0.0352-0.0467)]. The mean scutum CS comparison using Bonferroni test with 1000 permutations showed that almost all species had significant differences in mean scutum CS except for *W. ewingi lupella* and *W. ewingi ewingi*.

Perimeter was used as the size of scutum for outline-based GM analysis. Mean scutum perimeter length of *Walchia* spp. derived from outline-based GM analysis showed size differences (Fig. 2B, Table 2), which were consistent with landmark-based GM analysis. The largest scutum perimeter [mean (min-max) 0.1789 mm (0.1704-0.1841)] was observed in *W. ewingi ewingi*. *W. minuscuta* had the smallest scutum

Table 2
Statistical analyses of mean scutum centroid size derived from landmark-based GM and perimeter length from outline-based GM analysis of the four *Walchia* species [*Walchia ewingi* with 2 subspecies].

Scutum centroid size (Landmark-based GM analysis)				
Species	*N*	*Mean (Min-Max) (mm.)*	*S.D.*	*S.E.*
W. ewingi lupella	45	0.0553 (0.0513-0.0593)^{d}	0.0019	0.0003
W. ewingi ewingi	18	0.0540 (0.0523-0.0563)^{d}	0.0011	0.0003
W. alpestris	15	0.0478 (0.0451-0.0494)^{b}	0.0012	0.0003
W. kritochaeta	33	0.0516 (0.0470-0.0556)^{c}	0.0021	0.0004
W. minuscuta	29	0.0415 (0.0352-0.0467)^{a}	0.0026	0.0005
Scutum perimeter length (Outline-based GM analysis)				
W. ewingi lupella	41	0.1757 (0.1647-0.1874)^{c}	0.0055	0.0009
W. ewingi ewingi	16	0.1789 (0.1704-0.1841)^{c}	0.0038	0.0010
W. alpestris	15	0.1574 (0.1481-0.1681)^{b}	0.0048	0.0012
W. kritochaeta	26	0.1760 (0.1597-0.1875)^{c}	0.0076	0.0015
W. minuscuta	23	0.1399 (0.1256-0.1820)^{a}	0.0122	0.0026

Different superscript letters indicate significant differences between species at $p < 0.05$. Abbreviations: Min, minimum; Max, maximum; millimeters (converted from pixels); Mean, average centroid size; S.D., standard deviation; S.E., standard error.

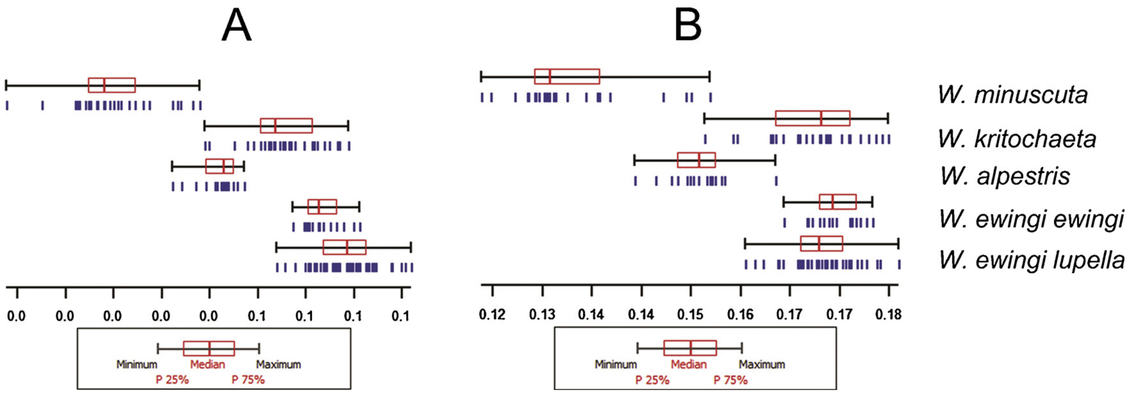

Fig. 2. Comparisons of the scutum size of mites from the genus *Walchia*.
Panel A: Box plot of the scutum centroid sizes (CS) using Landmark-based GM analysis reveals similar results to the perimeter of outline-based GM analysis (Panel B).
Note: Data is converted from pixels to mm, expressed as median with IQR (p25, p75), vertical blue bars under the boxes represent the size of the individual specimen.

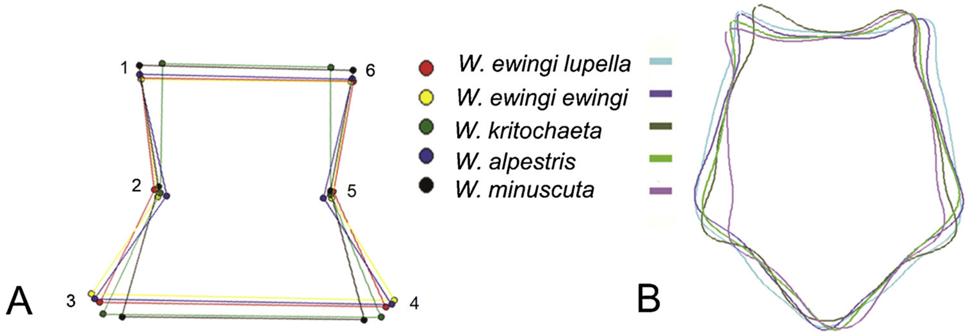

Fig. 3. Superposition of the mean shape landmark (A) and outline (B) configurations of *W. ewingi lupella*, *W. ewingi ewingi*, *W. alpestris*, *W. kritochaeta* and *W. minuscuta* (A). No amplification was applied.

perimeter length [mean (min-max) 0.1399 mm (0.1256-0.1820)]. *W. ewingi lupella*, *W. ewingi ewingi* and *W. kritochaeta* were not significantly different in mean size after Bonferroni test. However, other 2 species had absolutely significant difference in mean scutum size.

3.2. Scutum shape variation

Polygon superimposition of the mean landmark configuration showed scutum shape difference (Fig. 3A) without overlapping of scutum shape. In the same way, outline superimpositions of the scutum shape also showed contour differences (Fig. 3B) without overlapping. The factor map of the discriminant analysis of scutum shape showed species separation in both landmark-based (Fig. 4A) and outline-based GM (Fig. 4B). *W. alpestris* and *W. minuscuta* were clearly distinguished from other 2 species. Cross-validated classification is detailed in Table 3. Landmark-based GM provided better species separation than outline-based GM analysis ranging from 84%–100% and 43%–100%, respectively. The perfect scores were observed in *W. alpestris* provided by both techniques.

All pairwise Mahalanobis distances were different between species, which was statistically significant after Bonferroni test for landmark-based GM ($p < 0.001$) and outline-based GM analyses ($p < 0.001$), except for *W. kritochaeta* and *W. ewingi ewingi* ($p = 0.052$) (Table 4). These findings were visualized by the phenetic NJ trees based on the Procrustes distances. Both methods constructed the same pattern of NJ trees, which located *W. ewingi lupella, W. kritochaeta* and *W. ewingi ewingi* into the same cluster and separated *W. alpestris* and *W. minuscuta* (Fig. 5, A and B).

Table 3
Cross-validated classification for 4 *Walchia* species based on landmark-based and outline-based GM analyses of the scutum.

Species	classification score (N)	
	landmark-based GM	outline-based GM
W. ewingi lupella	88% (40 / 45)	85% (35/41)
W. ewingi ewingi	88% (16 / 18)	43% (7/16)
W. alpestris	100% (15 / 15)	100% (15/15)
W. kritochaeta	84% (28 / 33)	69% (18/26)
W. minuscuta	93% (27 / 29)	91% (21/23)

Values represent percent of correctly assigned individuals with corresponding details.

3.3. Genetic analysis based on partial COI gene

In total 20 partial *COI* gene sequences (637 bp length) were included in a phylogenetic tree; these included *W. ewingi lupella* (n = 9), *W. ewingi ewingi* (n = 2), *W. alpestris* (n = 2), *W. kritochaeta* (n = 5), *W. minuscuta* (n = 2) and *Blankaartia acuscutellaris* (n = 10), which served as the outgroup representatives.

The intraspecific divergence for *W. ewingi lupella* was 0.007 (range, 0.002-0.011), for *W. ewingi ewingi* 0.005, for *W. alpestris* 0.109, for *W. kritochaeta* 0.009 (range, 0.000-0.153), and for *W.* minuscuta 0.114 (range, 0.000-0.171). The four defined species were separated by an average interspecific divergence of 0.218 (0.026–0.323) (Table 5). Phylogenetic analysis revealed one distinct cluster: *W. kritochaeta* was separated from the cluster of *W. ewingi ewingi*, *W. ewingi lupella, W. alpestris*, and *W. minuscuta* (Fig. 6).

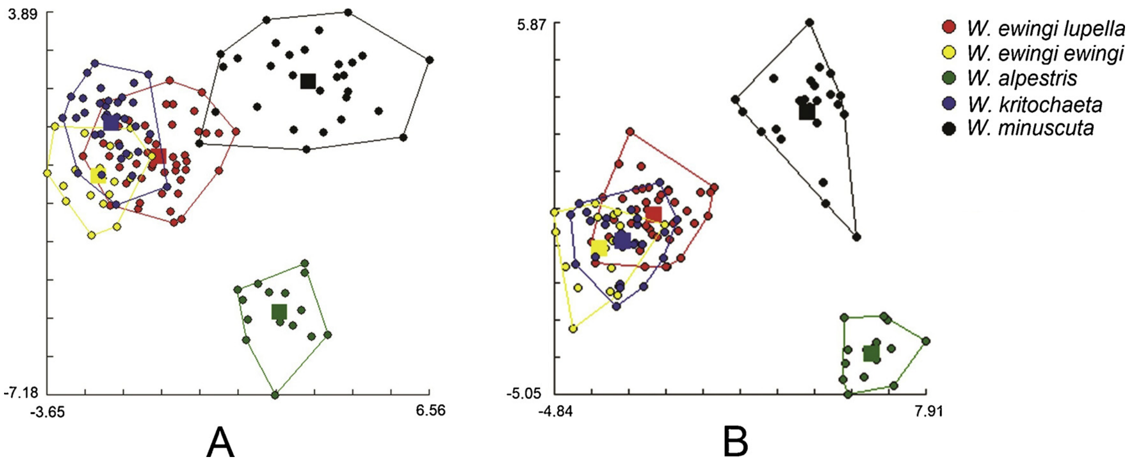

Fig. 4. Landmark-based DA (A) and outline-based DA (B). Factor map of the two discriminant factors (DFs) derived from final shape variables *W. ewingi lupella* (red), *W. ewingi ewingi* (yellow), *W. alpestris* (green), *W. kritochaeta* (blue) and *W. minuscuta* (black). Each point represents an individual sample. The horizontal axis is the first DF; the vertical axis is the second DF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4

Landmark-based and outline-based GM analysis, Mahalanobis distances between scutum shapes of *W. ewingi lupella*, *W. ewingi ewingi*, *W. alpestris*, *W. kritochaeta* and *W. minuscuta*.

Species	*W. ewingi lupella*		*W. ewingi ewingi*		*W. alpestris*		*W. kritochaeta*	
	LM	*OL*	*LM*	*OL*	*LM*	*OL*	*LM*	*OL*
W. ewingi lupella	0	0						
W. ewingi ewingi	2.49*	3.48*	0	0				
W. alpestris	6.29*	8.65*	6.62*	9.9 *	0	0		
W. kritochaeta	4.01*	3.3*	3.21*	1.66$^{\#}$	7.22*	9.82*	0	0
W. minuscuta	4.99*	6.47*	6.42*	8.21*	6.9*	7.43*	5.81*	7.46*

(* = p-value < 0.001, $^{\#}$ = p-value ≤0.05) (LM: landmark-based GM analysis, OL: outline-based GM analysis).

4. Discussion

In this study, we evaluated the potential benefits of scutum-based GM analysis in differentiating between the difficult-to-characterise mite species. We found that this method was useful in supporting or augmenting conventional morphology and improved characterisation and validation of genetic barcoding. We found that both landmark-based and outline-based GM methods allowed more in-depth discrimination between the notoriously difficult *Walchia* spp. that showed both genetic polymorphism (based on *COI* sequences) and morphological plasticity in a previous study (Kumlert et al., 2018).

4.1. The difficulties of conventional morphotyping

Morphological classification is considered the current gold standard for the characterization of chiggers and is especially relevant for the identification of scrub typhus vectors. Trombiculid mite species can have highly similar features, complicating their final identification at the species level to the point where sometimes only identification at the genus level is possible with confidence. This has implications on characterisation of vector-borne disease eco-epidemiology – especially where scrub typhus is highly endemic (Kingston et al., 2018; Wangrangsimakul et al., 2018). Some researchers have opted for alternative approaches that compare similarities and differences between species samples from different loci, like multidimensional scaling methods (Stekolnikov and Klimov, 2010). Conventional chigger mite morphometric measurements include measurements of scutum (AW, PW, SB, ASB, PSB, SD, AP), lengths of scutal setae (AM, AL, PL) and sensilla (S), lengths of idiosomal setae (H, D_{min}, D_{max}, V_{min}, V_{max}), lengths of legs (pa, pm, pp, Ip), and numbers of idiosomal setae (DS, VS, NDV) (Stekolnikov, 2018, 2013). To address cross-species morphological similarities, a statistical multivariate classification was developed to separate 3 closely related species of *Neotrombicula* (Stekolnikov and Klimov, 2010).

Our group recently developed an approach based on AF microscopy for the identification of key morphological features, which did not destroy the mite DNA, thus supporting the paired morphotyping and genotyping of trombiculid mites (Kumlert et al., 2018). However, we found evidence of both genetic and morphological plasticity in specimens attributed to the genus *Walchia*. These findings and the fact that important mite features may be lost or damaged – usually extremities or protruding head or mouth parts – highlighted the need of further improvement of the morphological identification process (Kumlert et al., 2018; Patsoula et al., 2006).

4.2. Morphotyping using GM approach

The scutum of chiggers, a dorsal shield of thick chitin, represents a central morphological characteristic for reliable identification at the genus level, as previously described in pictorial keys (Nadchatram and Dohany, 1974; Vercammen-Grandjean, 1968), and recently shown to be an equally relevant characteristic using AF imaging. AF microscopy exhibited excellent images of the scutum with well-delineated setae insertions to provide robust identification of chiggers at the subgenus level, even when setae or sensilla were damaged or missing (Kumlert et al., 2018). In this study we provide evidence that the scutum can be used for further differentiation beyond the genus level based on its outline and landmarks features using geometric morphometric analyses.

The classical morphological characteristics required for identification and distinction of *Walchia* spp. (other than scutum meaurements) are the number of coxa-III setae, which enable differentiation between

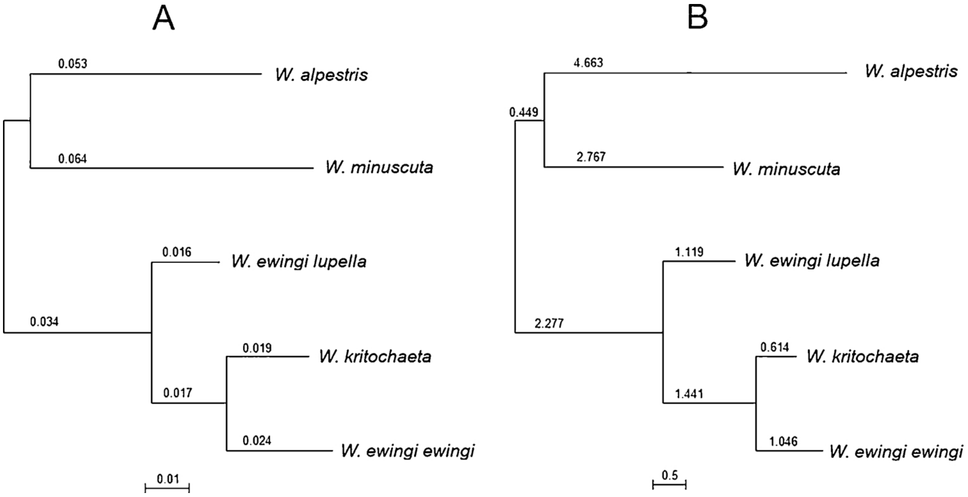

Fig. 5. NJ trees for landmark-based GM analysis (A) and outline-based GM analysis (B) of *W. minuscuta* and *W. alpestris*; *W. ewingi lupella, W. ewingi ewingi* and *W. kritochaeta*.

Table 5
The average interspecific divergence of *COI* sequences among chigger mite species.

Species	*W. ewingi lupella*	*W.ewingi ewingi*	*W. alpestris*	*W. minuscuta*	*W. kritochaeta*
W. ewingi lupella					
W. ewingi ewingi	0.126				
W. alpestris	0.186	0.188			
W. minuscuta	0.156	0.154	0.217		
W. kritochaeta	0.198	0.160	0.243	0.182	
Bl. acuscutellaris	0.294	0.254	0.323	0.308	0.282

W. ewingi lupella and *W. ewingi ewingi*. Normally *W. ewingi ewingi* has 3 coxal setae III, but the number can vary from 2 to 4 coxal setae III for different geographic regions (Traub and Evans, 1957; Vercammen-Grandjean, 1971). Despite detailed descriptions in classical pictorial keys, important limitations of this approach became apparent this is where additional GM data was able to support morphological identification in the case of disparate findings.

4.3. Paired morphotyping and genotyping

The improvement of paired morphotyping and genotyping addresses an important unresolved issue relating to a better understanding of scrub typhus transmission dynamics (Paris et al., 2013). With the recent availability of paired matched mite genotypes and morphotypes, we evaluated the accuracy of geometric morphometric techniques in comparison to DNA sequencing. DNA sequences based on 637 bp *COI* sequences accurately differentiated the *Walchia* spp. mites according to their morphotypes in the phylogenetic tree (Fig. 5). Differentiation based on the landmark and outline-based morphometric data resulted in comparable phylogenetic trees (Fig. 4), and both techniques unequivocally classified all 4 species of *Walchia*. Interestingly, the genetic approach based on *COI* genes demonstrated a higher resolution with evidence of possible sub-structuring below the species level in the *W. kritochaeta* cluster. Despite small sample sizes, there was preliminary evidence of both genetic polymorphism and morphological plasticity within these species. The specimens of *Walchia ewingi lupella* collected from *Bandicota indica* showed genetic diversity within a group of mites collected from a single host at a single location. It remains to be investigated whether this reflects potential cross-breeding of mites from different sub-populations or a limitation of the method (Takhampunya et al., 2018). This level of plasticity was not evident in the phenetic trees based on GM data, but some limitations were apparent in the PCA analyses; where the GM data was not able to separate clearly among *W. ewingi lupella, W. ewingi ewingi* and *W. kritochaeta* – albeit clearly

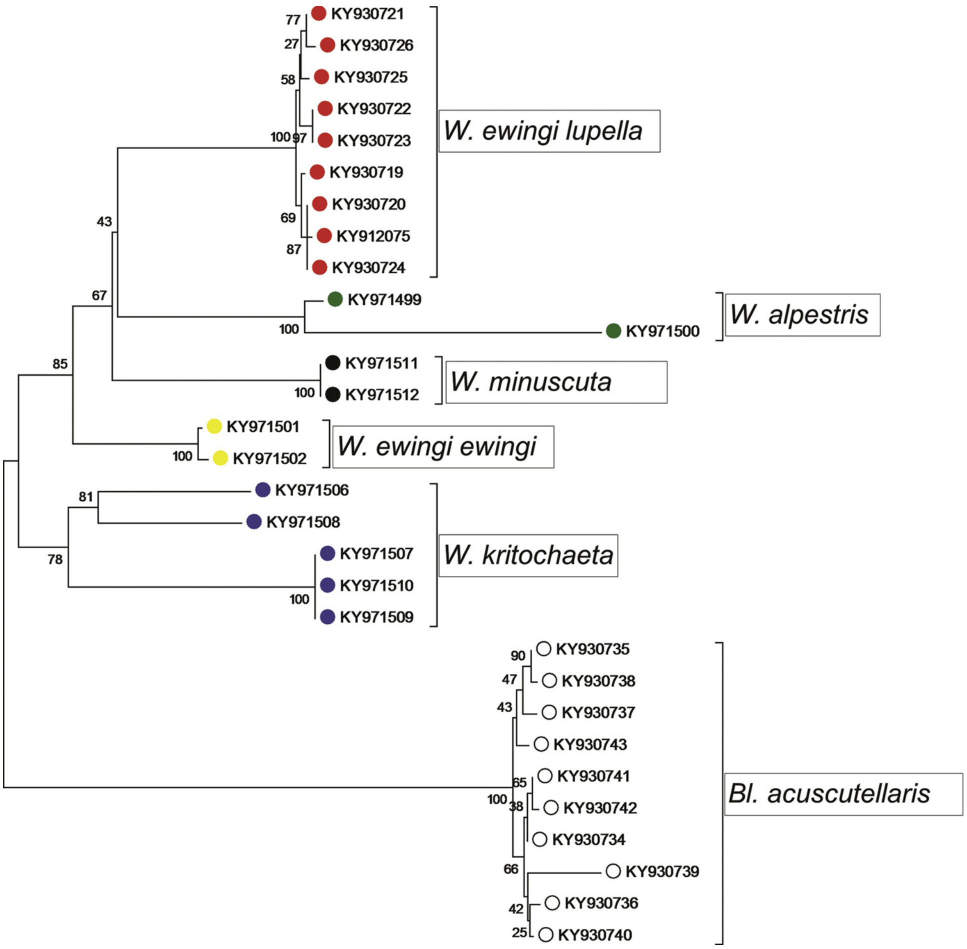

Fig. 6. Phylogenetic analysis of *COI* sequencing from *W. ewingi lupella* (9), *W. ewingi ewingi* (2), *W. alpestris* (2), *W. kritochaeta* (5) and *W. minuscuta* (2). Percentages of bootstrap values with 1000 replicates are based on maximum likelihood analysis. Scale bar represents 0.02 substitutions per site, *Blankaartia acuscutellaris* represent outgroups.

differentiating *W. alpestris* and *W. minuscuta* (Fig. 4).

It is interesting that *W. kritochaeta* was separated genetically from the *W. ewingi, W. alpestris* and *W. minuscuta* cluster, while morphologically *W. kritochaeta* grouped closely between *W. ewingi lupella* and *W. ewingi ewingi* (Figs. 5 and 6). In order to resolve these subtle differences, a broader research scope including host and environmental factors as well as gene-based classification scheme for comparative analyses is required.

For advancing further the much-needed characterisation of trombiculid mites, to generate a large data collection on paired morphogenotypic data for various geographic regions, the conventional morphological classification methods need to be improved as well – *i.e.* by incorporating geometric morphometric measurements.

The available keys by Traub and Evans (1957) to classify *Walchia* species show *W. alpestris* to have shorter pinnate hairs than the other 3 species; *W. minuscuta* is distinct from others by their smaller scutum size; only a single setae on coxa-III of *W. kritochaeta* is the characteristic that differentiates it from 2 closest taxa, *W. ewingi ewingi* and *W. ewingi lupella*. These 2 subspecies are differentiated by the number of coxa-III setae, length of sensilla, setae insertions and eye number (Traub and Evans, 1957). Since the variation in the number of coxal setae III is possible (Traub and Evans, 1957; Vercammen-Grandjean, 1971) and the number of eyes is not an unequivocal characteristic in every sample, it may possibly lead to wrong identification. These difficulties and discrepancies found in the classic morphological criteria could be resolved by the supplementary information that geometric morphometrics provides.

5. Conclusions

In summary, we found that geometric morphometrics are useful in supporting or augmenting the conventional morphological identification of trombiculid mites and contribute to improved characterisation and validation of genetic mite barcoding. The major advantage lies in the use of the easily-accessible, robust and usually intact scutum, to provide sufficient information for differentiation beyond the genus level into the species level.

We propose that future mite characterisation should include the use of AF microscopy to generate paired morphotypic and genotypic data (currently based on the *COI* gene) and that in cases of discrepant results, subsequent GM approaches using the same AF scutum images can easily be perfomed to resolve these difficulties. The collation of well-characterised mite morpho-genotype data into a broadly available database, will substantially facilitate the future identification of mites. Over time, the genotypic identification of trombiculid mites without the conventional fastidious morphotyping, could become the new gold standard. Until then, continued efforts should go towards the collecting of high-quality, standardised and prospective paired identification data on chigger mites. These data will subsequently support more in-depth investigations into aspects of the natural history of scrub typhus – the associations between mites, mammalian hosts, their endosymbionts and pathogens, and possibly the human dead-end hosts – and elucidate the genetic and morphometric variation found among them.

Acknowledgements

This study was supported in part by a scholarship granted to Rawadee Kumlert (FY2017) as a grant from the National Research Council of Thailand for doctoral degree students, a supportive research grant for the PhD thesis for Rawadee Kumlert from the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and a LKS Strategic Award (LG34) “The Natural Immune Response in Pediatric Scrub Typhus in Chiang Rai” from Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (DHP as PI). We would also thank Asst. Prof. Ronald Enrique Morales Vargas for sharing his knowledge on Geometric Morphometric methods and Mr. Tanawat Chaiphongpachara for his valuable guidance on the Geometric Morphometric program, and the “Intensive workshop for manuscript writing & proposal writing (17–19 October 2017)” supported by Faculty of Tropical Medicine and The Thailand Research Fund (TRF) for R. Kumlert.

References

Bureau of Epidemiology MoPH Thailand, 1980. Scrub Typhus. pp. 2010–2014.

Chaisiri, K., 2016. Molecular Ecology of Chigger Mites (Acari: Trombiculidae) and Associated Bacteria in Thailand. Dep. Infect. Biol. Inst. Infect. Glob. Heal. University of Liverpool, England.

Changbunjong, T., Sumruayphol, S., Weluwanarak, T., Ruangsittichai, J., Dujardin, J.P., 2016. Landmark and outline-based geometric morphometrics analysis of three *Stomoxys* flies (Diptera: muscidae). Folia Parasitol. (Praha) 63. https://doi.org/10.14411/fp.2016.037.

De La Riva, J., Le Pont, F., Ali, V., Matias, A., Mollinedo, S., Dujardin, J.P., 2001. Wing geometry as a tool for studying the *Lutzomyia longipalpis* (Diptera: Psychodidae) complex. Mem. Inst. Oswaldo Cruz 96, 1089–1094. https://doi.org/10.1590/S0074-02762001000800011.

Dujardin, J.-P., 2008. Morphometrics applied to medical entomology. Infect. Genet. Evol. 8, 875–890.

Dujardin, J.-P., Le Pont, F., 2000. Morphometrics of a neotropical sandfly subspecies, *Lutzomyia carrerai* thula. Comptes Rendus l’Académie des Sci. III-Sciences la Vie 323, 273–279.

Dujardin, J.-P.A.L., Kaba, D., Henry, A.B., 2010. The exchangeability of shape. BMC Res. Notes 3, 266.

Dujardin, J.P., Kaba, D., Solano, P., Dupraz, M., McCoy, K.D., Jaramillo-O, N., 2014. Outline-based morphometrics, an overlooked method in arthropod studies? Infect. Genet. Evol. 28, 704–714. https://doi.org/10.1016/j.meegid.2014.07.035.

Dujardin, J.P., Slice, D., 2007. Geometric morphometrics. Contributions to medical entomology. Encycl. Infect. Dis. Mod. Methodol. 435–447 Vol. Chapter 25.

Efron, B., 1983. Estimating the error rate of a prediction rule: improvement on cross-validation. J. Am. Stat. Assoc. 78, 316–331.

Fernandes, S., Kulkarni, S.M., 2003. Studies on the trombiculid mite fauna of India. Records of the zoological survey of India. Occas. Pap. 212, 1–539.

Jaramillo-O, N., Dujardin, J., Calle-Londoño, D., Fonseca-González, I., 2015. Geometric morphometrics for the taxonomy of 11 species of *Anopheles* (Nyssorhynchus) mosquitoes. Med. Vet. Entomol. 29, 26–36.

Josek, T., Allan, B.F., Alleyne, M., 2017. Morphometric analysis of chemoreception organ in male and female ticks (Acari: Ixodidae). J. Med. Entomol. 55, 547–552.

Kingston, H.W., Hossain, M., Leopold, S., Anantatat, T., Tanganuchitcharnchai, A., Sinha, I., Plewes, K., Maude, R.J., Chowdhury, M.A.H., Paul, S., Uddin, R.A.M.E., Siddiqui, M.A.N., Zahed, A.S., Abu Sayeed, A., Rahman, M.H., Barua, A., Uddin, M.J., Sattar, M.A., Dondorp, A.M., Blacksell, S.D., Day, N.P.J., Ghose, A., Hossain, A., Paris, D.H., 2018. Rickettsial illnesses as important causes of febrile illness in Chittagong, Bangladesh. Emerg. Infect. Dis. 24 (4). https://doi.org/10.3201/eid2404.170190.

Kuhl, F.P., Giardina, C.R., 1982. Elliptic Fourier features of a closed contour. Comput. Graph. Image Process. 18, 236–258.

Kumlert, R., Chaisiri, K., Anantatat, T., Stekolnikov, A.A., Morand, S., Prasartvit, A., Makepeace, B.L., Sungvornyothin, S., Paris, D.H., 2018. Autofluorescence microscopy for paired-matched morphological and molecular identification of individual chigger mites (Acari: Trombiculidae), the vectors of scrub typhus. PLoS One 13 e0193163.

Liu, Y., Yang, Z., Wu, Q., Sun, H., Meng, X., Peng, Z., Miao, Z., 1999. Isolation of *Rickettsia tsutsugamushi*from trombiculid mites (Acari: Trombiculidae) in Feixian county, Shandong province, China. Insect Sci. 6, 171–177.

Manly, B.F.J., 2004. Multivariate Statistical Methods: A Primer. CRC Press.

Nadchatram, M., Dohany, A.L., 1974. A pictorial key to the subfamilies, genera and subgenera of Southeast Asian chiggers (Acari, Prostigmata, Trombiculidae). Bull. Inst. Med. Res. Malaysia 16, 1–67.

Paris, D.H., Shelite, T.R., Day, N.P., Walker, D.H., 2013. Unresolved problems related to scrub typhus: a seriously neglected life-threatening disease. Am. J. Trop. Med. Hyg. 89 (2), 301–307.

Patsoula, E., Samanidou-Voyadjoglou, A., Spanakos, G., Kremastinou, J., Nasioulas, G., Vakalis, N.C., 2006. Molecular and morphological characterization of Aedes albopictus in northwestern Greece and differentiation from *Aedes cretinus* and *Aedes aegypti*. J. Med. Entomol. 43, 40–54.

Pretorius, E., Clarke, F.C., 2001. Geometric morphometric analysis of the male and female body shape of *Amblyomma gemma*, *A. variegatum and A. hebraeum*. Int. J. Acarol. 27, 271–279.

Prudhomme, J., Cassan, C., Hide, M., Toty, C., Rahola, N., Vergnes, B., Dujardin, J.-P., Alten, B., Sereno, D., Bañuls, A.-L., 2016. *Phlebotomus ariasi* (Diptera: Psychodidae) in the region of Roquedur (Gard, France): a geometric morphometrics approach. Parasit. Vectors 9, 578.

Rohlf, F.J., Slice, D., 1990. Extensions of the procrustes method for the optimal superimposition of landmarks. Syst. Zool. 39, 40. https://doi.org/10.2307/2992207.

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. https://doi.org/10.1016/j.meegid.2011.10.002.

Stekolnikov, A.A., 2013. (Acari: Trombiculidae) of the World. Zootaxa 3728, 1–173. https://doi.org/10.11646/zootaxa.3728.1.1.

Stekolnikov, A.A., 2018. Taxonomy and distribution of African chiggers (Acariformes, Trombiculidae). Eur. J. Taxon. 395, 1–233. https://doi.org/10.5852/ejt.2018.395.

Stekolnikov, A.A., Klimov, P.B., 2010. A revision of chiggers of the minuta species-group (Acari: trombiculidae: *neotrombicula* Hirst, 1925) using multivariate morphometrics. Syst. Parasitol. 77, 55–69. https://doi.org/10.1007/s11230-010-9258-7.

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. https://doi.org/10.1016/j.actatropica.2016.03.010.

Takhampunya, R., Korkusol, A., Promsathaporn, S., Tippayachai, B., Leepitakrat, S., Richards, A.L., Davidson, S.A., 2018. Heterogeneity of *Orientia tsutsugamushi* genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand. PLoS Negl. Trop. Dis. 16 (7), e0006632 12.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Traub, R., Evans, T.M., 1957. Malaysian parasites, XXVIII. Records and descriptions of chiggers of the subgenus *Walchia* Ewing, 1931, from Southeast Asia (Acarina, Trombiculidae). Stud. Inst. Med. Res. Malaya 28, 297–358.

Traub, R., Wisseman Jr, C.L., 1974. The ecology of chigger-borne rickettsiosis (scrub typhus). J. Med. Entomol. 11, 237–303.

Vargas, R.E.M., Phumala-Morales, N., Tsunoda, T., Apiwathnasorn, C., Dujardin, J.-P., 2013. The phenetic structure of Aedes albopictus. Infect. Genet. Evol. 13, 242–251.

Vercammen-Grandjean, P.H., 1971. Revision of the species *Walchia disparunguis* (Oudemans, 1929) and its group (Acarina, Trombiculidae). Opusc. Zool. (München) 115, 1–12.

Vercammen-Grandjean, P.H., 1968. The Chigger Mites of the Far East: Acarina: Trombiculidae & Leeuwenhoekiidae: An Illustrated Key and a Synopsis, Some New Tribes, Genera, and Subgenera. U. S. Army Medical Research and Development Command, Washington, D.C 135 pp.

Vercammen-Grandjean, P.H., Langston, R., 1976. The Chigger Mites of the World (Acarina: Trombiculidae et Leeuwenhoekiidae). Vol. III. *Leptotrombidium* Complex. George Williams Hooper Foundation, University of California, San Francisco 1061 pp.

Voltzit, O.V., Pavlinov, I.Y., 1996. Application of the methods of a geometrical morphometrics to the Ixodid ticks (Ixodidae) taxonomy. Parazitologiia 30, 292–301.

Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3, 294–299.

Wangrangsimakul, T., Althaus, T., Mukaka, M., Kantipong, P., Wuthiekanun, V., Chierakul, W., Blacksell, S.D., Day, N.P., Laongnualpanich, A., Paris, D.H., 2018. Causes of acute undifferentiated fever and the utility of biomarkers in Chiangrai, northern Thailand. PLoS Negl. Trop. Dis. 12 (5), e0006477 31.

Womersley, H., 1952. The scrub-typhus and scrub-itch mites (Trombiculidae, Acarina) of the Asiatic-Pacific region. Rec. South Aust. Museum 10, 1–435.

Yu, E.-S., Lin, S.-G., 1957. Study on the condition of natural infection with *R. Tsutsugamushi* among mites and domestic animals in Fukien. Acta Microbiol. Sin. 5, 425–432.