Spatial analysis of wing geometry in dengue vector mosquito, Aedes aegypti (L.) (Diptera: Culicidae), populations in Metropolitan Manila, Philippines

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Spatial analysis of wing geometry in dengue vector mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae), populations in Metropolitan Manila, Philippines

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ABSTRACT

Background & objectives: Aedes aegypti (L.) is an efficient vector for arboviral diseases such as dengue. The wings of *Ae. aegypti* has been extensively studied in order to investigate population heterogeneity and structure by utilizing a landmark based geometric morphometrics (GMs) approach. The aim of this study was to examine and assess the wing geometry of *Ae. aegypti* in Metropolitan Manila.

Methods: In total, 312 *Ae. aegypti* mosquitoes were collected from 98 sampling points using a mosquito light-trap from May 2014 to January 2015. A complete coverage of the wing was achieved by removing wing scales with chemical and physical treatment, leading to identification of 26 landmarks. Geometric morphometric analyses were employed and the spatial distance pattern was estimated using isolation by distance (IBD) and spatial autocorrelation (SA).

Results: The results of the GM analyses revealed population heterogeneity and structuring in *Ae. aegypti* populations for both sexes using principal component and canonical variate analyses respectively. Moreover, IBD and SA only detected significant spatial structure in male *Ae. aegypti* populations while female population structures were homogeneous throughout the geographical area.

Interpretation & conclusion: The newly modified wing preparation procedure was able to capture a complete coverage of the wings of *Ae. aegypti*, thus providing a stronger separation power for very close populations in an urban area. It is also noteworthy that the results of IBD and SA supported the findings of GM in the population structuring of male and female *Ae. aegypti*. The outcome of the study increases our understanding of the vector, which would be useful in developing effective control strategies.

Key words *Aedes aegypti*; dengue; geometric morphometrics; landmark; Philippines; wing shape

INTRODUCTION

The occurrence and dispersal of *Aedes aegypti* in urban communities have been a public health concern due to its role as an efficient vector of dengue, an important arthropod-borne viral disease[1]. Currently, dengue prevention is largely focused in controlling its principal vector, *Ae. aegypti*. Thus, understanding the population biology and spatial dispersal patterns are keys in controlling the vector because these are considered to be the major factors in the spread of the disease[2–5].

Several tools have been used in order to understand the spatial and dispersal patterns of *Ae. aegypti*. In the past decade, geometric morphometrics (GMs) has been utilized as an alternative technique to determine population structures using metric properties of size and shape of an organism's structure. It is considered as an effective, fast and low-cost approach that can infer recent population events[6]. Wings of insects, such as found in *Ae. aegypti,* are valuable structures in studying morphological variation because of its bidimensional rigid form[7–10]. The use of wings in this approach allows researchers to view metric changes in its properties which are directly linked to its function, *e.g.* either by flight or sexual signaling in *Ae. aegypti*[3,11]. Studies of *Ae. aegypti*'s wing geometry had been reported in many countries such as the United States [3], Colombia[3], Brazil[8], Thailand[10], Indonesia[12] and the Philippines[7]. These studies demonstrated homogeneity in wing shape and claimed that wings of *Ae. aegypti* are well-preserved or stabilized in spite of pressures from environmental changes or stress, founder effect or genetic drift and species competition[3, 8]. In

Indonesia[12] and the Philippines[7], intraspecific variation and morphological disparity were observed suggesting morphological divergence.

The procedure of GM studies in medically important insects has been properly outlined by Rohlf and Marcus[13]; and Zeldith *et al*[14]. An important step in GM is the acquisition of landmark (LM) data which is the basis for morphometric analysis. GM landmarks are points that can describe the shape of a structure and are categorized into three types (I, II and III)[15]. For the purposes of this study, only two categories were used, namely Type I, specific locally defined features (*e.g.* wing venation points) while Type II are points found in the minima or maxima of a curvature[14–15]. The GM emphasizes that a complete coverage of the structure is favourable to achieve a comprehensive LM analysis that may lead to additional insights of shape variation[16]. All *Ae. aegypti* GM studies, as to date, showed incomplete removal of wing scales and differing number of LM establishments from 13 to 20 LMs[3, 10]. The presence of wing scales for geometric morphometric study adds as a limiting factor for visualizing LMs, hence, can produce obscurity and error in LM establishment. The protocol by Lorenz and Suesdek[17] demonstrated the removal of wing scales by using either physical or chemical treatment prior to geometric LM acquisition. These methods produced high repeatability and reproducibility scores of LMs that lead to better visualization. In the present study, we used both treatments simultaneously and modified the exposure time of the chemical treatment. Our procedure showed a more complete coverage of the wing of *Ae. aegypti* wherein we identified 26 LMs.

Succeeding steps in LM data analysis use multivariate analyses to depict shape variation. In the present study, we included the use of isolation by distance (IBD)[18] and global spatial autocorrelation (SA)[19] in order to explore intraspecific variation under limited and geographical dispersal of the vector mosquito. It also allows to infer finer-scale and short term spatial distribution under a small geographic area. The use or application of these analyses in the metric properties of the wing shape of *Ae. aegypti* are not yet explored, however, such analysis is very limited in different organisms[20–22]. Hence, this may provide an insight in elucidating the observed morphometric variation.

The main objectives of the study were to examine and assess the wing shape variation and population structure of *Ae. aegypti* in Manila metro, Philippines. The highlights of the study were the development of a procedure to reveal more LMs and a complete coverage of wing shape.

MATERIAL & METHODS

Study site and mosquito sampling

Metropolitan Manila, a highly urbanized area, is the national capital region (NCR) of the Philippines[23] with an area of 636 km^2. It is located at the eastern shore of Manila Bay in Southwestern Luzon (14°50' N Latitude, 121°E Longitude), Philippines, Southeast Asia. NCR is composed of 17 cities and one municipality. It is further divided into four Districts comprising the different cities and municipality, namely Capital District (CD), Eastern Manila District (EMD), Northern Manila District (NM) and Southern Manila District (SMD) (Fig. 1).

A total of 312 adult *Ae. aegypti* (215 females and 97 males) were collected from May 2014 to January 2015 in 98 sampling sites. Minimum and maximum distance from each sampling site was about 0.5 and 43 km respectively. Commercially-available mosquito light-traps, Mosquito traps®, were used for the collection of mosquito samples. It utilizes the use of ultraviolet light wherein it serves as a

Fig. 1: Geographic map of Philippines (right) and its National Capital Region (NCR) also known as Metropolitan Manila (left) showing the boundaries of the administrative districts. Dots indicate sampling sites from a period of May 2014 to January 2015.

catalyst for the production of titanium dioxide (TO_2) coated in the funnel of the trap in order to lure adult mosquitoes. The trap also has a powerful vacuum action fan that traps the insects inside the cage and prevents it from flying out until the mosquito dies. These adult traps were placed in the outdoor premises of each sampling site for 3–5 days. Individual mosquito samples were labeled with all pertinent information and assigned in geographic populations, namely NMCD, EMD and SMD for comparative purposes. Mosquitoes identified as *Ae. aegypti*[24] were separated as males and females and individually kept for wing morphometric study.

Wing preparation and geometric data acquisition

Both wings of an individual adult mosquito were detached from the thorax by carefully cutting from the base of attachment. Removal of wing scales was done following the procedure of Lorenz and Suesdek[17] with some modifications. The presence of wing scales is considered as limiting factor for visualizing landmarks, hence, producing error in LM establishment (Fig. 2a). The protocol of Lorenz and Suesdek[17] separately utilized a physical and chemical treatment of the wings. In contrast, the present study combined the use of chemical and physical treatment by treating the detached wing with 10% potassium hydroxide (KOH) for 10–15 min and gently removing the scales with a fine paint brush. After removal of wing scales, the treated wing was mounted onto a glass slide and cover slip with Aquatex®. Images of the wing were captured using Nikon SMZ800 dissecting microscope with camera in a 3.5x magnification. The wing preparation of this study was more efficient as compared to the chemical method of Lorenz and Suesdek[17]. In their paper, they recommended a soaking time of 12 h in 10% KOH. This made the wing veins transparent and LM points not visible. The Fig. 2b shows an example of a wing with transparent wing venation when soaked for only six hours. Hence, they added a staining reagent to make it visible for LM acquisition.

The present study was able to identify 26 LM (Fig. 2c). LMs 1–19 and 25 are landmarks established from reports of Sendaydiego *et al*[7] and Jirakanjanakit *et al*[10] while LMs 20–24 and 26 are our additional landmarks to allow overall wing shape analysis (Supplemental Table 1). LMs 20 and 26 are considered to be Type II LMs while the remaining LMs are Type I. The coordinates of 26 LM of each individual wing were obtained using TpsDig version 1.4[25]. Moreover, further analysis was done to compare the separation power in population structuring of the different sets of LMs, namely 20[10], 18[7–8];

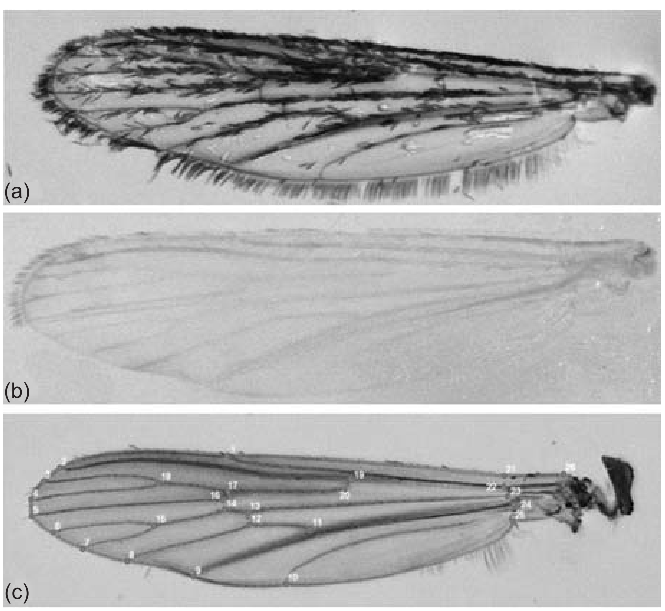

Fig. 2: Wing of *Aedes aegpyti:* (a) With scales obscuring wing venation intersection; (b) Chemically treated wing with 10% KOH for 6 h showing nearly transparent wing venation; and (c) Chemically treated wing with 10% KOH for 10–15 min plus physical treatment of wings by gently brushing with a fine paint brush. Numbers indicate the 26 landmarks (detailed in Supplemental Table 1).

Supplemental Table 1. Corresponding description and location of the wing and landmark positions in *Ae. aygypti*

Landmark	Type	Location
1	I	Intersection of costa
2	I	Distal end of the radius
3	I	Radial branch 2
4	I	Radial branch 3
5	I	Distal end of the radius branches 4 and 5
6	I	Distal end of M1 and 2
7	I	Distal end of M3 and 4
8	I	Distal end of cubital vein 1
9	I	Distal end of cubital vein 2
10	I	Anal vein
11	I	Origin of cubital 1
12	I	Midpoint branch of cubital 3
13	I	Medio-cubital cross vein
14	I	Midpoint branch of medial vein
15	I	Radio-sectoral vein
16	I	Radio-medial cross vein
17	I	Midpoint branch of radial vein
18	I	Origin of radius branches 2 and 3
19	I	Midpoint branch of radius 1
20	II	Curvature of the radial cross vein
21	I	Humeral cross vein of costal margin
22	I	Humeral cross vein of subcostal margin
23	I	Basal end of media (near distal plate)
24	I	Basal end of cubitus (near to proximal plate)
25	I	Jugal fold
26	II	Minimum curvature of the basal end of costa

15[26], and 13[3] LMs based on the mosquitoes' group clusters and geographic location using Mahalanobis distance.

Geometric morphometric analysis

The measurement error was tested by comparing three sets of digitization and determined by the repeatability index as described by Arnqvist and Martensson[27]. This was initially performed by landmarking independently 151 individual's wing images by three researchers. Repeatability index was computed using Model II One-way ANOVA and Pearson's correlation[28] from the three measurements done by each independent researcher for all individual's wings. The left wing was prepared for the geometric data acquisition, however if the left wing was damaged and not usable, the right wing was used. In all, 232 mosquito individuals with intact left and right wings were checked for wing symmetry by Pearson's correlation.

In assessing the wing size variation, the isometric estimator known as centroid size[15] was computed from the LMs coordinates using MorphoJ[29]. The centroid sizes between the sexes based on geographic groups and group clusters were subjected to comparative analysis using parametric ANOVA by the PAST software[30].

In assessing wing shape variation, shape variables were obtained using the generalised least-squares procrustes superimposition algorithm[14]. Principal component analysis (partial wraps)[15] explored the shape variation in both sexes. Canonical variate analysis (CVA) was done with 10,000 permutations to test for pairwise distances between geographic populations and group clusters defined by unweighted pair group method with arithmetic mean (UPGMA) (see Results) in both sexes. To visually aid in exploring intraspecific wing shape variation, the thin-plate spline interpolation[14] was used. The procrustes distance was utilized to produce a dendrogram using the UPGMA algorithm. All the analyses were achieved using MorphoJ and PAST software.

To determine if there is an effect of size on the shape of the wing, a test of allometry was performed. This was tested for both males and females using multivariate regression of log- transformed centroid size and the shape variation based from the Procrustes coordinates with 10,000 permutations for testing of significance. The test was performed using MorphoJ.

Isolation by distance and spatial autocorrelation

Two matrices were used; (a) the geographic distances (in km) between mosquito samples were calculated using the Vincenty's formulae on Microsoft Excel; and (b) Proximity matrix calculated from UPGMA algorithm. These two matrices were subjected to Mantel's test with 10,000 permutations and SA with 10,000 permutations and Bootstrap. Both analysis yielded correlation coefficient with a test for a significant relationship by random permutation. Results of the permutation were considered significant at 5% level. Furthermore, the SA coefficient in this analysis is closely related to Moran's I and provided a measure of similarity between individuals whose geographic separation falls within a specified distance class[31]. A correlogram was produced with four distance classes at 11 km interval. All these analyses were performed using genetic analysis in Excel (GenAlEx) version 6.3[32]. Additionally, the means of the proximity matrix calculated from UPGMA algorithm were compared for male and female *Ae. aegypti* using the Mann-Whitney test. This analysis was done using the PAST software.

RESULTS

Measurement error and landmark comparison

Table 1 shows the different number of LM establishments employed in different studies that investigated the wing of *Ae. aegypti*. The 26 LMs has the highest measurement for the centroid size because of the complete coverage of the wing. Moreover, the 26 LMs possessed the highest Mahalanobis distance among other LMs. It infers that since 26 LMs was able to show the overall shape of the wing, this set of LMs might constitute in separating groups effectively for intraspecific studies.

Table 1. Comparison of different LMs establishments of centroid sizes (mean ± centroid sizes) and Mahalanobis distances between group clusters of male and female *Ae. aegypti*

Characteristics	Landmarks (LMs)				
	26	20	18	15	13
Centroid sizes of male (Mean ± SD)	1225 ± 153	787 ± 102	603 ± 81	733 ± 93	485 ± 65
Centroid sizes of female (Mean ± SD)	1388 ± 194	900 ± 130	698 ± 106	860 ± 125	559 ± 85
Mahalanobis distance between male group clusters using CVA	3.82	3.51	2.90	3.01	2.81
Mahalanobis distance between female group clusters using CVA	6.74	5.69	4.62	5.17	4.13

LMs—Landmarks; SD—Standard deviation; CVA—Canonical variate analysis.

Hence, we used the 26 LMs for geometric morphometric analysis. The comparison of the independent measurements by three researchers for the same 151 individual images showed a good agreement for the centroid size (r = 0.98) and shape variability (r = 0.99, 0.99 for x, y coordinates respectively). Pearson correlation of the size and shape for each individual of the independent measurements ranged from 0.99–1 indicating a very high or perfect relationship. Hence, independent LM establishments were nearly the same. There have been reports in different mosquito species that exhibit asymmetry in right and left wings[33–35]. Symmetry of the right and left wing confirmed identical pattern based on Pearson's correlation (r = 1, n = 232).

Size and shape analysis of wing of male and female Ae. aegypti

The average centroid sizes of the wing of female and male *Ae. aegypti* were significantly different from each other (t = 9.75, $p < 0.001$) with 7.23 mm and 7.10 mm, respectively. The variation of wing shape explained by the first two PCA axes were 53.39% in females (PC1 = 44.73%, PC2 = 8.66%) and 55.08% in males (PC1 = 46.95%, PC2 = 8.13%). PCA scatter plots of wings from male and female *Ae. aegypti* showed two potential group clusters (Figs. 3a and 4a). Constructed UPGMA trees of male and female *Ae. aegypti* wings confirmed the distinction of the two group clusters for both sexes (data not shown). This in turn, provided another basis for grouping mosquito samples into "group clusters". Separation of group clusters was more prominent in males (MGC1 and MGC2) as compared to females (FGC1 and FGC2).

Further, analysis revealed that centroid sizes of group clusters of both sexes showed significant differences (p = 0.0001 in males, $p < 0.0001$ in females). Shape variation using CVA showed significant differences in group clusters for both male (Mahalanobis distance = 6.74, $p \leq 0.0001$) and female (Mahalanobis distance = 3.82, $p \leq 0.001$) mosquito samples. The test of allometry showed that the contribution of size to the shape of the wings was statistically significant for both males and females ($p \leq 0.001$). The proportion of the variance of shape that could be explained by size was found to be 5.06 and 2.72% for females and males, respectively. The percentages presented from the test of allometry were nearly the same as reported in previous studies. Such finding is considered to be relatively weak to let the wing size influence the wing shape variation[3, 8]. Furthermore, wireframe graphs showed broader wing shape for clusters FG1, FG2 and MG2 except for MG1 where it has a slender shape (Figs. 3b and 4b). Morphospace transformation grids of selected individuals in both sexes showed contraction of the center wing venations, LMs 11–20, and partial contraction of wing vein base, LMs 21–25 (Figs. 3a and 4a).

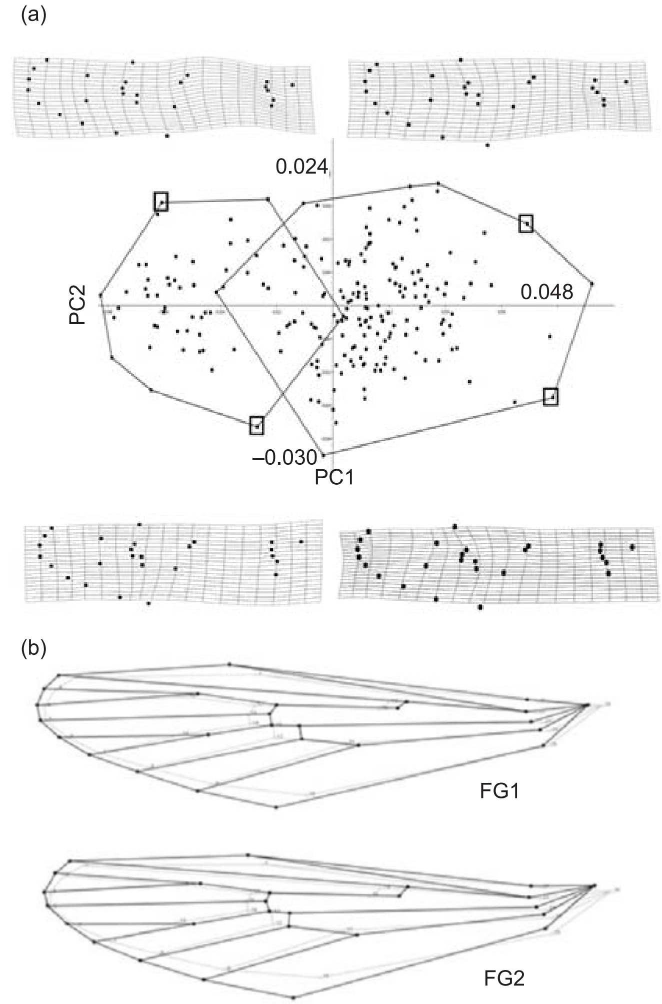

Fig. 3: Wing shape analysis of female (♀) *Ae. aegypti*— (a) Scatter plot of principal component (PC1 and PC2) analysis. Convex hulls indicate group clusters based on UPGMA tree of female groups (FG1 and FG2). Morphospace of selected individual transformation grids along the PCA scatter plot. Boxes with points on the middle indicate selected individual for thin plate spline diagram; and (b) Wireframe diagram of FG1 and FG2. Light-coloured wireframe represents mean wing shape of female *Ae. aegypti* while dark-coloured wireframe represents generated principal component of FG1 or FG2.

Size and shape analysis based on geographical locations

Centroid wing sizes among the three[3] different geographical groups showed no significant difference for male (F = 0.39, df = 2, $p = 0.68$) and female (F = 1.95, df = 2, $p = 0.15$) *Ae. aegypti* (Table 2). Shape variation based on CVA revealed significant differentiation among all male

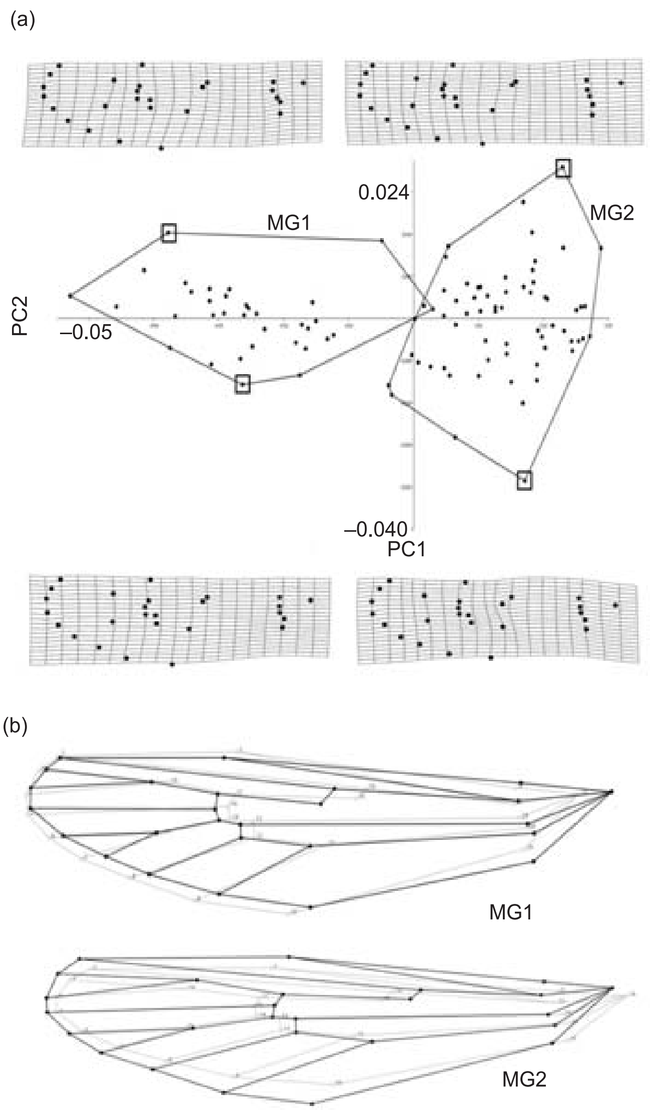

Fig. 4: Wing shape analysis of male (♂) *Ae. aegypti*— (a) Scatter plot of principal component (PC1 and PC2) analysis. Convex hulls indicate group clusters based on UPGMA tree of male groups (MG1 and MG2). Morphospace of selected individual transformation grids along the PCA scatter plot. Boxes with points on the middle indicate selected individual for thin plate spline diagram; and (b) Wireframe diagram of MG1 and MG2. Light-coloured wireframe represents wing mean shape of male *Ae. aegypti* while dark-coloured wireframe represents generated principal component of MG1 or MG2.

Ae. aegypti geographical groups, however female *Ae. aegypti* geographical groups showed significant differentiation between EMD from NM+CD and SMD (Fig. 4a and Table 2). Moreover, Mahalanobis distances among geographical groups were observed to be farther in male *Ae. aegypti* (2.06–2.98) as compared to female *Ae. aegypti* (1.27–1.64). Furthermore, Mantel tests showed significant but weak isolation by distance in males (r = 0.014, p = 0.005) but no significant isolation by distance in females (r = 0.0002, p = 0.27). Likewise, Mann-

Table 2. Statistical summary of mean centroid size (CS), Mahalanobis distance (MD), and isolation by distance (r), of male and female *Ae. aegypti* wings and their corresponding *p*-values

Characteristics	Male		Female	
Mean centroid sizes	CS	*p*-value	CS	*p*-value
Group 1	1172.81	0.0001	1178.29	<0.0001
Group 2	1321.96		1451.86	
NMCD	1219.29	0.15	1390.74	0.68
EMD	1186.78		1374.87	
SMD	1253.43		1402.03	
Mahalanobis distance	MD	*p*-value	MD	*p*-value
Group 1 – Group 2	6.74	<0.0001	3.82	<0.0001
NMCD – EMD	2.98	<0.0001	1.64	<0.0001
NMCD – SMD	2.17	0.0069	1.27	0.44
EMD – SMD	2.06	0.0001	1.29	0.03
Isolation by distance	r	*p*-value	r	*p*-value
	0.014	0.005	0.0002	0.27
Mean proximity index	Mean		Mean	*p*-value
	1.93		1.90	0.01

NMCD—Northern Manila and Capital District; EMD—Eastern Manila District; SMD—Southern Manila District; *p*-values are significant.

Whitney test indicated significant mean differences of the proximity matrix between male (1.93) than female *Ae. aegypti* (1.90) (U = 5.03 x 10^7 , p = 0.01). Correlograms from SA showed male *Ae. aegypti* have limited spatial distance (~22 km) while female *Ae. aegypti* have widespread spatial distance throughout the metropolitan area (Fig. 5).

DISCUSSION

Population heterogeneity of two group clusters was determined based on PCA and UPGMA in both sexes of *Ae. aegypti* populations. Most of the observed wing variability was found in wing contractions from the center (LMs 11–20) towards the base (LMs 21–26) based on thin-plate splines. This observation can be attributed to the wing's mechanical rigidity and stability[7]. Thin-plate splines also showed that the males have steeper base tip giving a shorter wingspan while females showed a broader base resulting to a longer and wider wingspan (LMs 1–10). These observed differences between males and females might affect their flight performance in relation to their body size and dispersal differences[36]. In the study, wing sizes of female *Ae. aegpyti* were observed to be larger

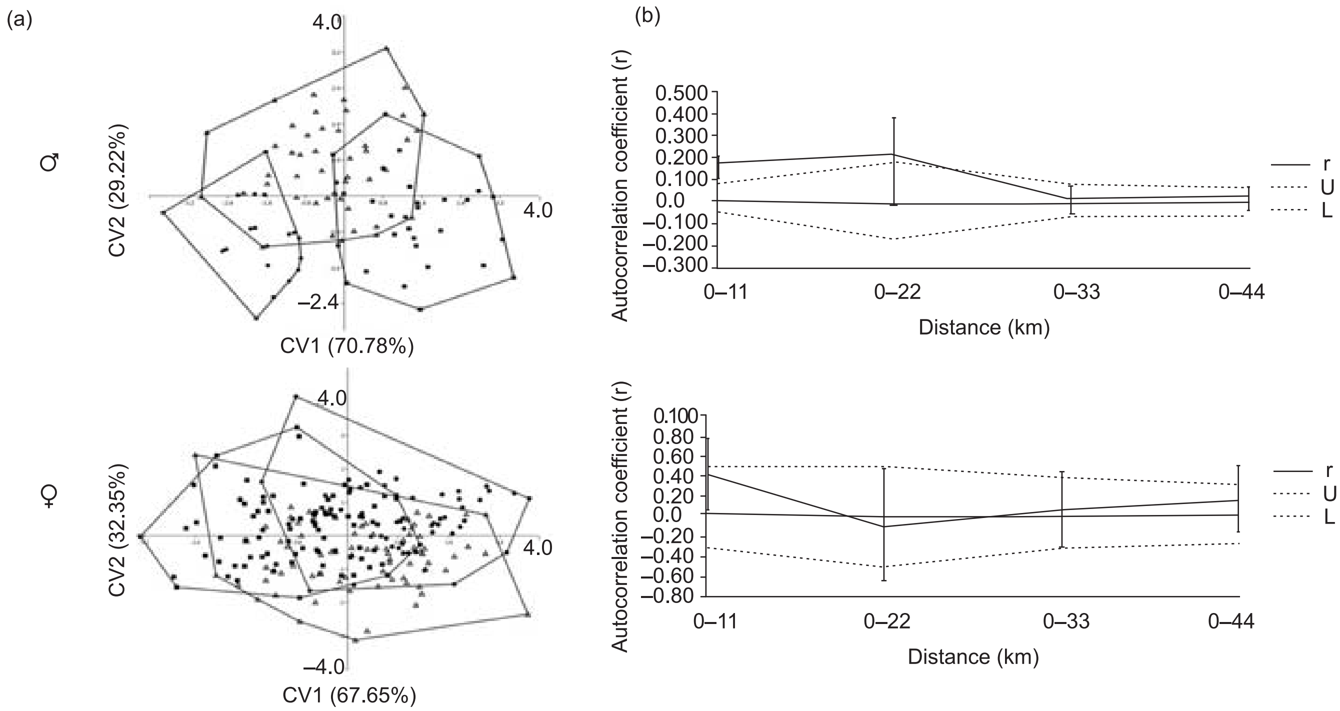

Fig. 5: Wing shape analysis of *Aedes aegypti* of male (♂) and female (♀) based on the geography; NMCD (Northern Manila and Capital Districts of Metro Manila), EMD (Eastern Manila District); and SMD (Southern Manila District)— (a) Canonical variate analysis (CVA) scatter plot with confidence ellipses for each geographic group (■ = NMCD ● = EMD, and △ = SMD); and (b) Correlogram of spatial autocorrelation showing the coefficient (r) up to 44 km with 11 km intervals. U and L are upper and lower confidence interval limit respectively.

compared to males. Wing and body-size sexual dimorphism have been reported to be present in *Ae. aegypti*[36]. These variations in the wing shape and size of *Ae. aegypti* populations may be attributed to the environmental heterogeneity of the geographical area causing possible phenotypic plasticity and local adaptation. The assumption is that Metropolitan Manila can exhibit high potential environmental heterogeneity. Thus, the size and shape of *Ae. aegypti* wings can be affected by ecological factors such as availability of food[37], successive generations[10] and climatic patterns[8, 38]. Assuming that these ecological factors were present, it might have allowed and supported the vector mosquito's adaptability, shaping its various biological aspects[39], notably its wing size and shape. Moreover, the present method (sampling and LMs) also contributed in finding heterogeneous populations. The preparation of the adult wing was essential in obtaining the complete coverage of the wing. The present study and Lorenz and Suesdek's protocol[17] underscore the need of such preparation in order to establish landmarks' efficiency and effectiveness. The methodology in preparing an individual wing only takes about 30 min depending on the experience of the researcher. Hence, this preparation is not very tasking nor difficult to be operated with larger sample sizes. The LM selection used both Type I and II LMs to capture the overall shape of the wing. Hingst-Zaher and Moraes[40] emphasized the use of as many LMs as possible or combination of different LM types which can allow better visualization and clear discrimination of groups. In this study, the 26 LMs showed the highest Mahalanobis distance that indicates a better measure of group separation. Strauss[41] discussed the essential of the Mahalanobis distance for geometric morphometrics in leading to different degrees of structuring.

Population structuring was fairly distinct in male *Ae. aegypti* as compared to female *Ae. aegypti* based on CVA. It was noteworthy that the use of broad and finer spatial pattern analyses (IBD and SA) confirmed these results wherein IBD was relatively weak for both sexes, however, significant with male *Ae. aegypti.* Furthermore, correlograms of the SA analysis corroborate to the scatter plot of CVA by showing that female *Ae. aegpyti* may have widespread spatial pattern indicating a population structure that may be homogenized in nature as compared to its male counterparts. These observed differences between both sexes can be reflective towards their dispersal ability. It was further suggested that dispersal of *Ae. aegypti* requires assistance in order to move from one area to the other possibly by transportation *via* human activities[42–44]. Many studies had speculated that this may be attributed to numerous road connections[45], continuous trade and migration[46]. Metropolitan Manila is a highly urbanized and populous area in Philippines wherein there are many commercial establishments and road connections between cities. Thus, eggs, immature stages or adults may be carried through this long-range dispersal.

CONCLUSION

The study was able to reveal population heterogeneity and structuring of *Ae. aegypti* in a small geographic area. The wing preparation procedure of this present study was instrumental to the complete coverage of the wing to be used in GM analysis. The metric properties of the wing were also utilized for fine-scale spatial distribution pattern analysis wherein its findings supported the results of the GM analysis. The spatial distribution pattern provided additional insight on why population heterogeneity and structuring were observed in both sexes of *Ae. aegypti.*

Conflict of interest

The authors declare that there is no conflict of interest in this study.

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